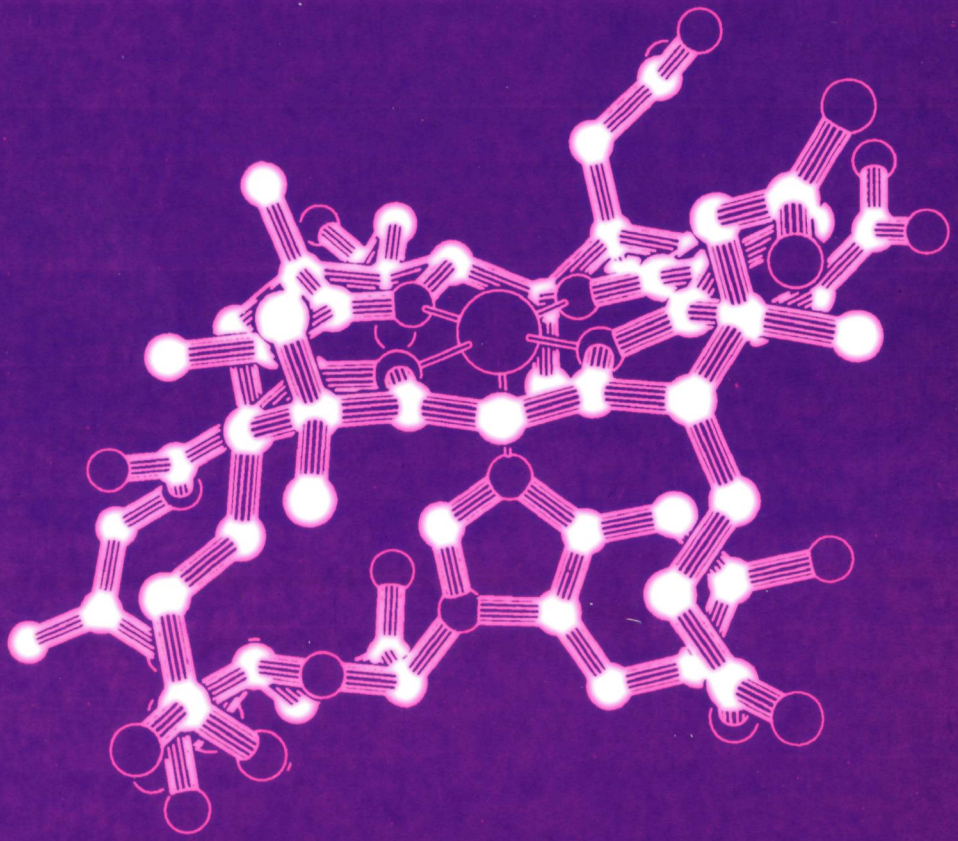
Methanol-dependent methyl transfer reactions in *Methanosarcina barkeri*



Piet J.H. Daas

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Cover: 3-Dimensional representation of the base coordinated form of 5-hydroxybenzimidazolylcob(II)amide. The structure was derived from the crystalline structure of adenosylcobalamin and plotted with the program PLUTON (A.L. Spek, unpublished) at the CAOS/CAMM center, University of Nijmegen, The Netherlands.

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Methanol-dependent methyl transfer reactions in *Methanosarcina barkeri*

EEN WETENSCHAPPELIJKE PROEVE
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PETRUS JOHANNES HENRICUS DAAS

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The investigations described in this thesis were carried out at the Department of Microbiology, University of Nijmegen, The Netherlands (Chapter 2-7) and at the Department of Biochemistry, Agricultural University, Wageningen, The Netherlands (Chapter 3,4 and 6).

Contents

"Imagination is more important than knowledge.
Knowledge is limited. Imagination encircles the world."
Albert Einstein: *On Science*.
In: Viereck, G.S., (1929) What Life Means to Einstein.
The Saturday Evening Post, Oct. 26, p. 117

General introduction

Abbreviations used: AdoMet, S-adenosylmethionine; B_{12}-DMBI, cobalamin, 5,6-dimethylbenzimidazolylcobamide; B_{12}-HBI, 5-hydroxybenzimidazolylcobamide; CODH, carbon monoxide dehydrogenase; Ni/Fe-S, nickel/iron-sulfur component of CODH; Co/Fe-S, corrinoid/iron-sulfur component of CODH; DEAE, diethylaminoethyl; DMBI, 5,6-dimethylbenzimidazole; EPR, electron paramagnetic resonance; F_{430}, a nickel-tetrapyrrole derivative; H_4MPT, 5,6,7,8-tetrahydromethanopterin; H_4SPT, 5,6,7,8-tetrahydrosarcinapterin; HBI, 5-hydroxybenzimidazole; HS-CoA, coenzyme A; HS-CoM, coenzyme M, 2-mercaptoethanesulfonic acid; CH_3-S-CoM, methylated coenzyme M, 2-(methylthio)ethanesulfonic acid; HS-HTP, 7-mercaptoheptanoylthreonine phosphate; CoM-S-S-HTP, the heterodisulfide of HS-CoM and HS-HTP; MAP, methyltransferase activation protein; MFR, methanofuran; MT_1, methanol:5-hydroxybenzimidazolylcobamide methyltransferase; MT_2, Co-methyl-5-hydroxybenzimidazolylcobamide: HS-CoM methyltransferase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate

1.1. Methanogenic archaea

The production of methane as a major catabolic product is unique to methanogens, that differ in a number of aspects from other micro-organisms. Phylogenetically, methanogens belong to the Archaea [84,85], the third grouping next to Eucarya and Bacteria (Fig. 1). Archaea are characterized by a number of specific properties. The membranes contain lipids composed of isoprenoids ether-linked to glycerol or other carbohydrates [16]. In addition, the cell walls are composed of proteins, glycoproteins, or polysaccharides [35], and lack peptidoglycan containing muramic acid [34]. Furthermore, archaea contain DNA-dependent RNA polymerases that are insensitive to the antibiotics rifampicin and streptolydigine [88]. In the archaea two major lineages can be distinguished (Fig. 1). One comprises most of the extremely thermophilic, sulfur-dependent micro-organisms for which the name crenarchaeota is suggested [85]. It is a physiologically

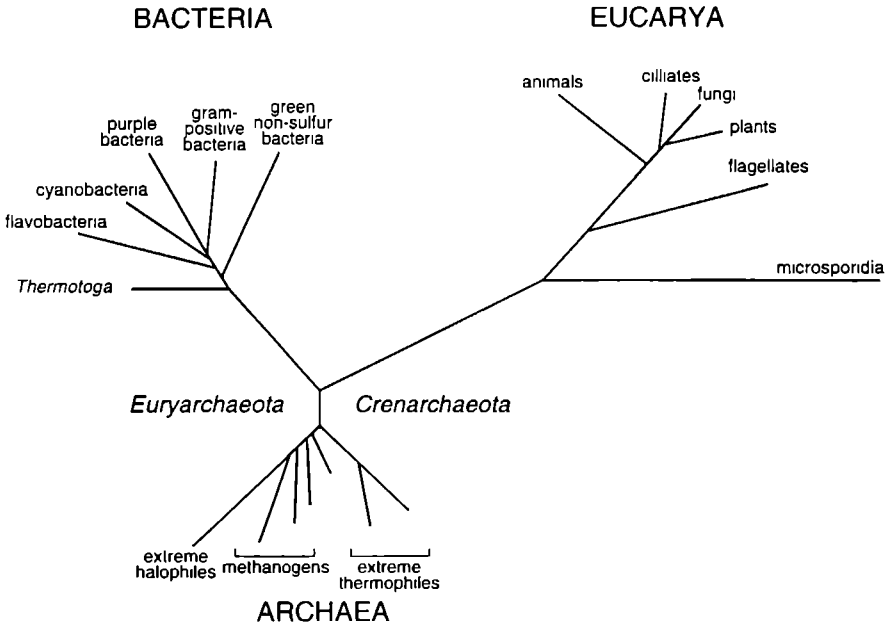

Fig. 1. Universal phylogenetic tree showing the three domains. Branching order and branch lengths are based on 16S rRNA sequence comparison [84,85].

Table 1. *Conversion of methanogenic substrates*

					ΔG° (kJ/mol CH_4)
CO_2	+	$4H_2$	⟶	$CH_4 + 2H_2O$	-130 4
4HCOOH			⟶	$CH_4 + 3CO_2 + 2H_2O$	-119 5
4CO	+	$2H_2O$	⟶	$CH_4 + 3CO_2$	-185 5
$2CH_3CH_2OH$	+	CO_2	⟶	$CH_4 + 2CH_3COOH + H_2O$	-116[a]
CH_3COO	+	H^+	⟶	$CH_4 + CO_2$	-36 0
$4CH_3OH$			⟶	$3CH_4 + CO_2 + 2H_2O$	-106
CH_3OH	+	H_2	⟶	$CH_4 + H_2O$	112 5
$4CH_3NH_2$	+	$2H_2O$	⟶	$3CH_4 + CO_2 + 4NH_3$	76 7
$2(CH_3)_2NH$	+	$2H_2O$	⟶	$3CH_4 + CO_2 + 2NH_3$	74 8
$4(CH_3)_3N$	+	$6H_2O$	⟶	$9CH_4 + 3CO_2 + 4NH_3$	75 8
$4CH_3SH$	+	$2H_2O$	⟶	$3CH_4 + CO_2 + 4H_2S$	-51
$2(CH_3)S$	+	$2H_2O$	⟶	$3CH_4 + CO_2 + 2H_2S$	-52 2

[a] Other short-chain alcohols like isopropanol may also be utilized

relatively homogeneous group, whose niches are entirely thermophilic [84] The other group is phenotypically heterogeneous, comprising the extreme halophiles, the sulfate-reducing species (the genus *Archeoglobus*), two types of thermophiles (the genus *Thermoplasma* and the *Thermococcus-Pyrococcus* group), and three methanogenic lineages (the *Methanococcales*, the *Methanobacteriales*, and the *Methanomicrobiales*) For this group the name euryarchaeota is proposed [85]

The methane-producing archaea can truly be termed cosmopolitan Methanogens exhibit extreme habitat diversity Species have been isolated from virtually every habitat in which anaerobic biodegradation of organic compounds occurs, including freshwater and marine sediments, digestive and intestinal tracts of animals and insects, and anaerobic waste digesters [89] Additional isolates have also been obtained from extreme environments such as geothermal springs and both shallow and deep-sea hydrothermal vents [89] Morphologically, methanogens are also diverse and occur as rods, cocci, packets of cocci (sarcinas), filaments, spirilla, and angular plates [58] The physiologically most salient feature is the extreme catabolic specialization of methanogens As a group they can only use a small number of simple compounds for methanogenesis and growth (Table 1) Many methanogens use only one or two substrates A major consequence of this substrate specialization is that in most anaerobic habitats, methanogens are dependent on other organisms for their substrates Therefore, a food web of interacting groups of anaerobes is required to convert most

organic matter to methane. During the last 5 years, methanogens were isolated that where able to convert substrates like methanethiol, dimethylsulfide, tetramethylammonium, pyruvate, and secondary alcohols [48,67, 83]. The latter alcohols are solely used as electron donors. The broadest versatility in substrate use is displayed by the methyl-group utilizing methanogens, such as *Methanosarcina barkeri*. This organism is able to grow on H_2/CO_2, methanol, acetate, CO, and mono-, di- and trimethyl-amine. The conversion of methanol by *M. barkeri* strain MS (DSM 800) [13] is studied in this thesis.

1.2. Corrinoids

Since the pioneering studies of Barker and associates [43], methanogens are known to contain considerable amounts of corrinoids, compounds that are structurally related to vitamin B_{12} [42,51]. To discuss their role in methanogenesis from methanol, a short general introduction to the corrinoids seems appropriate.

Almost 50 years ago, the red cobalt complex vitamin B_{12} was firstly isolated as the antipernicious factor present in liver. By 1956 it was structurally characterized as a porphyrin-like nucleotide-containing complex, in which pyrroles are linked by three methine bridges and one direct bond [18]. This so-called corrin [90] contains a cobalt coordinated to the nitrogen atoms of the pyrroles (Fig. 2). The cobalt atom may occur in three redox states, Co(III), Co(II), and Co(I), which display quite different chemical properties. Co(III) is six-coordinated by four equatorial nitrogens of the tetrapyrroles and by two axial ligands. In vitamin B_{12} the lower or α-ligand is 5,6-dimethylbenzimidazole, whereas the upper or β-ligand is a cyanide group (Fig. 2). Corrinoids in which the base is bound to the cobalt atom are named "base-on". When coordination is absent or replaced by an exogenous ligand the designation "base-off" is used. One-electron reduction of Co(III) results in a pentacoordinated Co(II) corrinoid, which has only one axial ligand. Reduction of Co(II) results in the formation of the square-planar, four-coordinated Co(I) corrinoid, one of the strongest nucleophiles in nature [18]. No α- or β-ligands are present. Oxidoreductive conversions between the three redox states of cobalt are of key importance in the biochemistry of corrinoids [59].

The various corrinoids isolated over the years usually differ in their α-ligand (Fig. 2) [25]. The most abundant corrinoid encountered in nature, called cobalamin, contains 5,6-dimethylbenzimidazole as its base [25]. Methanogens do not contain cobalamins, but instead three

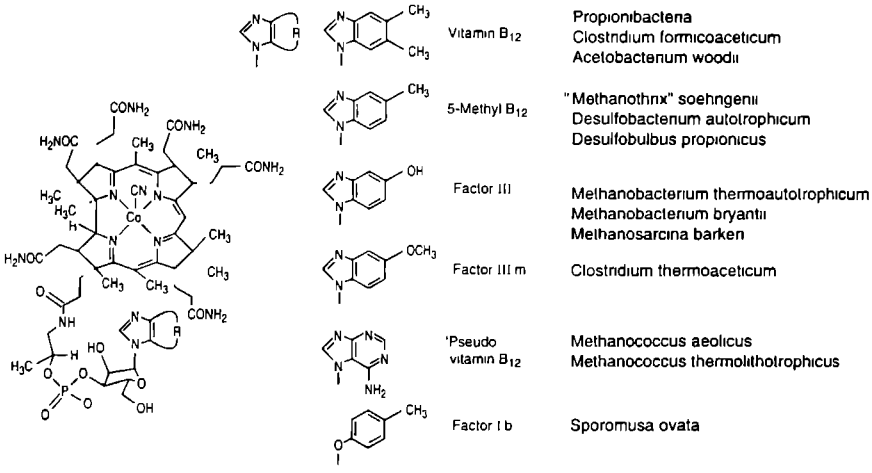

Fig. 2. Structure of corrinoids from bacterial origin showing the variability of the nucleotide portion.

other types of cobamides, base-containing corrinoids, have been found: factor III, pseudo-vitamin B_{12} and 5-methylbenzimidazolylcobamide. Factor III, which contains 5-hydroxybenzimidazole (HBI) as a base, is the prevalent cobamide found in the *Methanobacteriales* and the *Methanomicrobiales*. Its presence seems to be restricted to the methanogens [65]. Since the term factor III is also used to denominate an intermediate in cobyrinic acid biosynthesis, the abbreviation B_{12}-HBI is preferred [51]. Pseudo-vitamin B_{12}, containing adenine as a base, is the predominant cobamide of the *Methanococcales* though it is also found in Bacteria [65]. 5-Methylbenzimidazolylcobamide was isolated from *"Methanothrix" (Methanosaeta) soehngenii*, and this corrinoid is present in non-methanogens as well [41]. Obviously, cobamides of different structure occur in methanogens. Whether these variations in base composition affect the respective reactions of the cobamides, is not clear. It is notable, however, that vitamin B_{12} could be substituted for B_{12}-HBI in *Methanobacterium thermoautotrophicum* without affecting the growth rate [66].

Over the years several β-ligands have been described, including water, CN^-, OH^-, NO_2^-, SO_3^- [25,50], but only two have been shown to perform a coenzyme function, viz. methyl-B_{12} and 5-deoxyadenosyl-B_{12} [18]. The latter derivative, also known as coenzyme B_{12}, serves as a cofactor in various enzymatic reactions in which a hydrogen atom is displaced by a substituent on an adjacent carbon atom [2]. The C-C

rearrangement reactions proceed via a radical reaction induced by the protein-bound coenzyme [45]. As yet, this type of B_{12}-mediated reaction has not been encountered in methanogens and will therefore not be considered further. The methylated corrinoids are catalysts in biological methyl transfer reactions [44,59] that will be discussed below in some detail.

1.3. Biochemistry of methanogenesis

The results of the investigations on the biochemistry of methanogenesis have been thoroughly reviewed during the past decade [7,21, 22,36,69,82]. In this process, three basic metabolic pathways can be distinguished: the pathway from H_2/CO_2, from acetate, and from methanol. Methanogenic processes using other substrates are less well studied but seem to have many properties in common with these basic pathways [81]. In the following sections an overview will be given of the methanogenic metabolism of *M. barkeri*, notably methanogenesis from methanol. Methane production from acetate, H_2/CO_2, methylamines, and methylsulfides will only be briefly discussed with emphasis on the methyltransferase reactions involved.

1.3.1. Methanol reduction to methane

In 1938 Schnellen [54] obtained a pure culture of a sarcina-like methanogen, which he named *Methanosarcina barkeri* [13] and which was able to grow at the expense of methanol. From that time on, many attempts were made to elucidate the pathway by which methanol was reduced to methane. After the discovery that methylcobalamin could serve as a precursor for methanogenesis in extracts of *M. barkeri*, Blaylock and Stadtman [10] suggested that methanol fermentation might involve an enzyme-catalyzed transfer of the methyl group from methanol to cobalamin, followed by a reductive methylation to methane. Later they reported the enzyme-catalyzed formation of methylcobalamin from cob(I)alamin and methanol [11], and the system involved was resolved into four components [9,12]: a high-molecular weight corrinoid-containing enzyme, a ferredoxin-like compound, a protein with unknown function, and a heat- and acid-stable cofactor. The formation of methylcobalamin from methanol and electrochemically prepared cob(I)alamin required the presence of those four components as well as ATP and H_2. Another indication of the possible

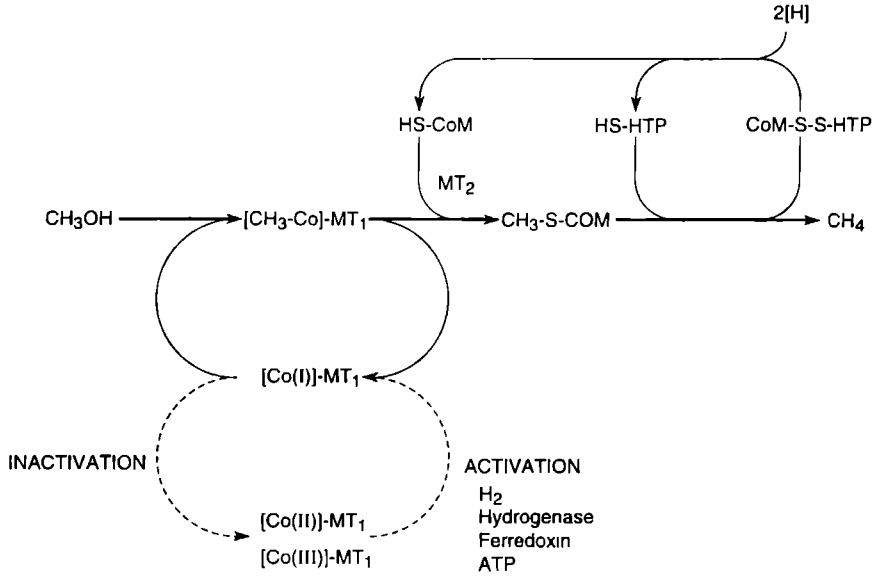

Fig. 3. Scheme of methanogenesis from methanol The cycle of activation-inactivation of methanol 5-hydroxybenzimidazolylcobamide methyltransferase (MT_1) is indicated by the dashed lines [Co(III)], [Co(II)], and [Co(I)] represent the various oxidation states of the cobalt of the corrinoid prosthetic groups of MT_1 MT_2, Co-methyl-5-hydroxybenzimidazolylcobamide coenzyme M methyltransferase, HS-CoM, coenzyme M, HS-HTP, 7-mercaptoheptanoylthreonine phosphate

involvement of corrinoids was the presence of rather copious amounts of B_{12}-HBI. In *M. barkeri* grown on methanol 4 1 nmol of corrinoid per milligram of cell dry weight was found [42]. After the discovery of coenzyme M (HS-CoM) and its central role as methyl carrier in methanogenesis, the role of B_{12} in the process became uncertain [55]. Studies on the corrinoid protein of *M. barkeri* continued, though quite some doubt remained [86] The work of van der Meijden et al. [72] definitely established a role for B_{12}-HBI in the conversion of methanol. Today, the role of corrinoids in methanogenesis is, finally, fully appreciated.

A scheme of the reactions involved in the conversion of methanol to methane in *M. barkeri* is presented in Fig 3. Reduction of methanol to methane proceeds via methylated coenzyme M (CH_3-S-CoM) [57], which is synthesized from methanol and 2-mercaptoethanesulfonic acid (HS-CoM) by the combined action of two methyltransferases [73]. The first methyltransferase, methanol:B_{12}-HBI methyltransferase (MT_1),

contains a corrinoid which is methylated by methanol, when it is present in its fully reduced Co(I) state. Next, the methyl group is transferred to HS-CoM by Co-methyl-5-hydroxybenzimidazolyl-cobamide:HS-CoM methyltransferase (MT_2). Both enzymes are insensitive to 2-bromoethanesulfonic acid, the typical inhibitor of the CH_3-S-CoM reductase system.

MT_1 has been purified from *M. barkeri* by a factor 5.7, as determined from the increase of the specific corrinoid content [76]. Nevertheless, the enzyme preparation was over 90% pure, which indicates MT_1 comprises about 15% of the soluble protein in the cell-extract. Purification required strict anoxic conditions and, even then, major loss in activity occurred. Native MT_1 showed an apparent molecular mass of 122 kDa and an $\alpha_2\beta$ configuration with two subunits of 34 kDa and one subunit of 53 kDa [76]. Per molecule of protein 3.4 molecules of B_{12}-HBI were found. The corrinoid was tightly, but not covalently bound to the holoenzyme, and it was completely lost upon dissociation of the enzyme into its subunits by treatment with SDS and 2-mercaptoethanol [76]. For stability, the enzyme required the presence of divalent cations, preferably Mg^{2+}. MT_1 is only active if the corrinoids are present in the fully reduced Co(I) state [75]. In the presence of viologen dyes, flavins, or oxygen oxidation of the reduced corrinoids readily occurs. Yet, reactivation is possible by the combined action of ATP and a reducing system consisting of H_2, hydrogenase, and ferredoxin [74,77]. ATP is required in only catalytic amounts. Its role remained, however, unknown [56,72,76,78]. GTP, CTP and, to some extent, UTP were able to substitute for ATP in the resolved system [76]. In crude extracts, hydrogen could be replaced by CO [76] and pyruvate plus coenzyme A (HS-CoA) [74], but not by the powerful reducing agent titanium(III)-citrate [78]. Addition of a protein fraction which was eluted just in front of MT_1 during a first DEAE-cellulose purification step, stimulated the overall methyltransferase reaction when added together with hydrogenase and ferredoxin [76]. The fraction contained at least one protein component, called component S. Activity was sensitive to boiling and oxygen. The protein involved was not further characterized [68].

As a result of the MT_1 reaction, an enzyme containing a firmly bound methyl-corrinoid is obtained [75]. Methylated MT_1 and non-protein-bound methylcobalamin both are substrates of the second methyltransferase MT_2 [73]. The protein is highly specific for HS-CoM as methyl group acceptor [73,87]. MT_2 is insensitive to oxygen and is composed of a single polypeptide with a molecular mass of 38 kDa [87]. In *M. barkeri* two isoenzymes are present that differ in electrophoretic and immunological properties and that can be separated by hydroxylapatite

chromatography [27]. The N-terminal amino acid sequences of the isoenzymes are only 55% similar [87]. Isoenzyme I predominates in methanol-grown cells (about 90%), whereas isoenzyme II is mainly present (about 80%) in cells grown on H_2/CO_2, trimethylamine or acetate [27,87]. For the MT_2 isoenzyme I a function in the conversion of methanol and for the MT_2 isoenzyme II a function in the conversion of trimethylamine has been suggested [87].

In the final step of methanogenesis CH_3-S-CoM is reduced to methane by the methylreductase system. This system has been extensively studied in *M. thermoautotrophicum* [70]. In this organism it consists of four enzymes or enzyme complexes catalyzing the following reactions. (i) The reduction of CH_3-S-CoM by 7-mercaptoheptanoylthreonine phosphate (HS-HTP) which results in the generation of methane and the heterodisulfide of HS-CoM and HS-HTP (CoM-S-S-HTP). (ii) The

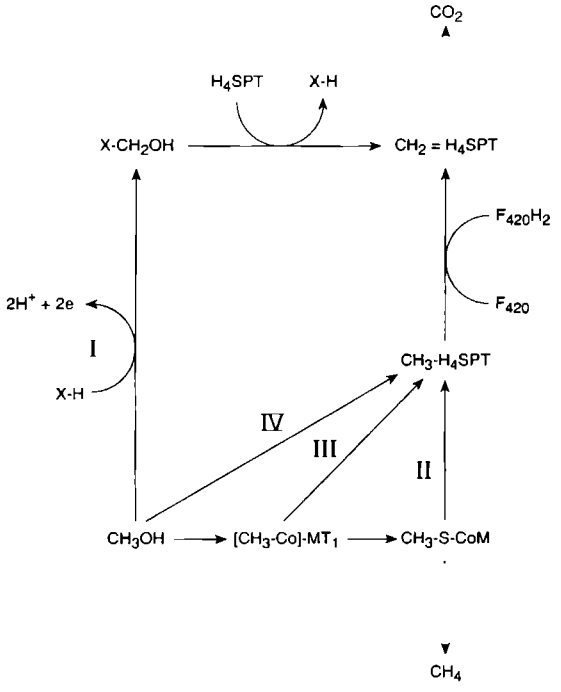

Fig. 4. Alternative pathways for the methanol methyl group oxidation. Roman numbers next to arrows indicate the different routes proposed in the text. H_4SPT, 5,6,7,8-tetrahydrosarcinapterin; F_{420}, a 8-hydroxy-5-deazaflavin derivative; X, unknown one-carbon carrier suggested by Blaut and Gottschalk [8].

reduction of a still unknown electron acceptor by hydrogenase. (iii) Transfer of the electrons of the unknown electron acceptor to a still unknown electron donor. (iv) Reduction of CoM-S-S-HTP by the unknown electron donor. Reaction (iv) and probably also reaction (iii) play a role in energy conservation [46]. The enzyme methylcoenzyme M reductase catalyzes the reductive demethylation of CH_3-S-CoM, reaction (i), and contains, as prosthetic group, 2 molecules of a nickel-tetrapyrrole called F_{430}. In cell-free and resolved systems methylcoenzyme M reductase is generally almost completely inactive. The enzyme can be reactivated to a few percent of the *in vivo* activity in an H_2- and ATP-dependent enzyme-catalyzed reaction [30]. The activation mechanism is poorly understood. Since active methylcoenzyme M reductase contains F_{430} in the highly reduced Ni(I) state [53] activation might imply the reduction of as-isolated Ni(II) into Ni(I).

1.3.2. Methanol oxidation to CO_2

In absence of hydrogen, *M. barkeri* oxidizes part of the methanol to CO_2 to generate the reducing equivalents needed for the reduction of the remainder of the methanol to methane. The oxidation route of methanol is not fully understood. Early experiments with [^{14}C]methanol revealed incorporation of label in "yellow fluorescent compound" as one of the intermediates in methanol oxidation [15]. Following its structural elucidation the compound is now known as 5,10-methenyl-5,6,7,8-tetrahydrosarcinapterin (methenyl-H_4SPT) [71] (see below). Experiments with whole cells of *M. barkeri* and *Methanosarcina mazei* strain Gö1 conclusively showed that a reaction between methanol and the formal redox level of formaldehyde is driven by a sodium-motive force [46]. The reaction (sequence) remains to be established and only a number of possible alternatives may be formulated (Fig. 4). One alternative (I) is the oxidation of methanol by use of a methanol dehydrogenase and an unknown one-carbon carrier [8]. In this case methanol would enter the oxidation pathway at the level of methylene-H_4SPT. However, the presence of methanol dehydrogenase has never been demonstrated. Other alternatives could be the MT_1-dependent activation of methanol (III) or CH_3-S-CoM synthesis (II), followed by the methyl group transfer to tetrahydrosarcinapterin (H_4SPT) (see below) or a direct methylation of H_4SPT by methanol catalyzed by a novel methyltransferase (IV) [37,46]. The oxidation of methyl-H_4SPT then follows the reversed CO_2 reduction route discussed in the next section.

1.3.3. Methanogenesis from H_2 and CO_2

The conversion of CO_2 to methane can formally be considered to proceed through the stages of formate, formaldehyde, and methanol. During this process the carbon atom remains bound to three different C_1-carriers (Fig. 5). In the first step carbon dioxide is reduced and bound to the first carrier, named methanofuran (MFR), yielding formylmethanofuran. Hereafter, the formyl group is transferred to the next C_1-carrier 5,6,7,8-tetrahydrosarcinapterin (H_4SPT). This compound is a structural analog of tetrahydrofolate and is used as a one-carbon carrier at the formyl, formaldehyde, and methyl level. In *M. thermoautotrophicum* 5,6,7,8-tetrahydromethanopterin (H_4MPT) is used, which is nearly identical to H_4SPT except for the absence of one glutamyl group in the sidechain [71]. 5-Formyl-H_4SPT is subsequently dehydrated to yield 5,10-methenyl-H_4SPT, which is reduced via 5,10-methylene-H_4SPT to 5-methyl-H_4SPT .

The transfer of the methyl group of methyl-H_4SPT to HS-CoM is catalyzed by an integral membrane protein [23] and proceeds in a two-step process in which methyl-B_{12}-HBI is an intermediate [49,79]. The enzyme complex catalyzing the overall reaction has been isolated from *M. thermoautotrophicum* strain Marburg and ΔH [26,38]. The former protein had an apparent molecular mass of 670 kDa and was composed of seven different subunits of 34, 28, 24, 23, 21, 13, and 12 kDa. It contained 7.6 mol B_{12}-HBI, 37 mol non-heme iron, and 34 mol acid-labile sulfur per mol of enzyme. The corrinoid was bound to the 23-kDa polypeptide [26]. Comparison of the N-terminal amino acid sequences of the subunits to the DNA-sequence of a 3.5-kb transcription unit of the *M. thermoautotrophicum* strain Marburg chromosome revealed that the 21-kDa, 23-kDa, and 24-kDa polypeptides constitute an integral part of the enzyme [63]. No such evidence is available for the four other polypeptides. However, the preparation was inactive when one of these four polypeptides was missing [26]. The purified enzyme from *M. thermoautotrophicum* strain ΔH was isolated as a 100-kDa protein, according to non-denaturating polyacrylamide gel electrophoresis (PAGE), and contained 0.2 mol B_{12}-HBI per mol of protein. When the protein was boiled in sample buffer and analyzed with SDS-PAGE polypeptides of 35, 33, and 31 kDa were observed [38]. However, Gärtner et al. [26] demonstrated that the polypeptide pattern of the purified methyltransferase drastically changed by this treatment. The enzyme of *M. barkeri* has not been purified so far.

The methyl-H_4SPT:HS-CoM methyltransferase reaction is dependent on reducing conditions and is stimulated by ATP [79]. In cell-free

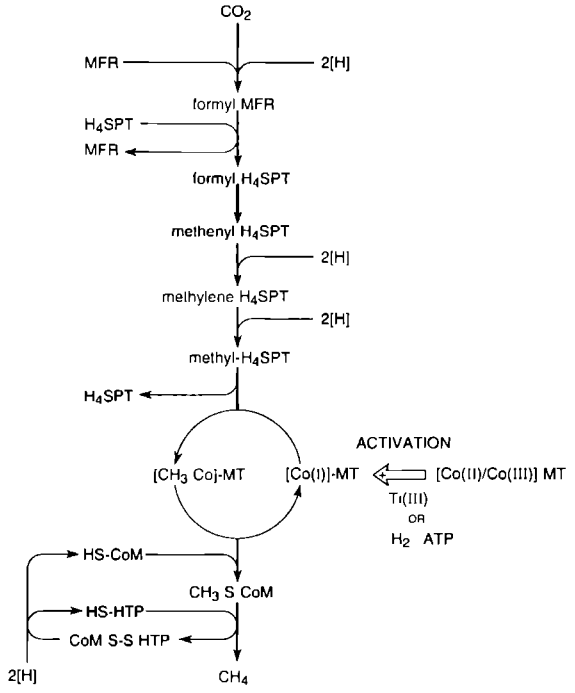

Fig. 5. Scheme of methanogenesis from H_2 and CO_2 in *Methanosarcina barkeri*. The activation of methyl-H_4SPT: HS-CoM methyltransferase (MT), indicated by the open arrow, is accomplished by H_2 and ATP that can be replaced by titanium(III)citrate MFR, methanofuran

extracts of *M. thermoautotrophicum* stimulation is accomplished by the combined action of ATP and CoM-S-S-HTP [39,40]. Ti(III)citrate is able to substitute for these requirements [26,38,40,79]. Ti(III)citrate, reducing conditions and ATP are all involved in generating the Co(I) form of the corrinoid [23,40,79]. Hereafter, the protein is able to accept the methyl group of methyl-H_4MPT [23,38,79]. The precise function of ATP in this process is unclear.

The standard free energy change ($\Delta G^{\circ\prime}$) associated with the methyl-H_4SPT:HS-CoM methyltransferase reaction is -29.7 kJ/mol [36]. At first it was considered unlikely that this would be a site for energy conservation, since there was no precedent for energy conservation being coupled to a methyl group transfer reaction. Experiments with inverted vesicles of *M. mazei* strain Göl, however, clearly demonstrated that this reaction was coupled with the generation of a sodium motive force [6].

Since the transfer of the methyl group of methyl-H_4MPT to hydroxycobalamin was also associated with sodium translocation [5], it was assumed that the transfer reaction from methyl-H_4MPT to the membrane-bound corrinoid is the actual exergonic step promoting the extrusion of sodium. The sodium-motive force thus generated can be converted into a proton-motive force by means of a sodium-proton antiporter, thus allowing synthesis of ATP by proton-translocation [46]. Immunological evidence exists that the purified proteins catalyzing the methyl-H_4MPT:HS-CoM methyltransferase reaction are identical to the membrane-bound corrinoid proteins of unknown function already described in 1986 [62]. Interestingly, membrane-associated corrinoids are found in all methanogens tested so far [14].

As a result of the methyl-H_4MPT:HS-CoM methyltransferase reaction CH_3-S-CoM is produced which is reduced to methane as described above (section 1.3.1.).

1.3.4. Methanogenesis from acetate

Conversion of acetate by methanogens proceeds by the cleavage of the molecule into a methyl group and a carbonyl group (Fig. 6). The methyl group is reduced to methane with electrons derived from oxidation of the carbonyl group to CO_2. The overall $\Delta G^{\circ\prime}$ is -36 kJ/mol for this reaction which is nearly equal to the energy required for synthesis of one molecule of ATP under standard conditions (+32 kJ/mol). This makes acetate a poor substrate for growth. Methanogenesis from acetate starts by converting acetate to acetyl-CoA at the expense of one ATP per acetate [21]. This is a considerable investment considering the small amount of energy available for ATP synthesis. Hereafter, acetyl-CoA is converted by the carbon monoxide dehydrogenase (CODH) complex. Although the name suggest otherwise the primary function of CODH is cleavage of acetate. The enzyme complex not only catalyzes cleavage of the C-C and C-S bond of acetyl-CoA, it also oxidizes CO to CO_2 (hence its name) and transfers the methyl group to H_4SPT (Fig. 6).

In *Methanosarcina thermophila* the CODH complex consists of five subunits which can be resolved into two enzymic components: a 200-kDa CO-oxidizing nickel/iron-sulfur (Ni/Fe-S) component which contains 89 and 19 kDa subunits, and a 100 kDa corrinoid/iron-sulfur (Co/Fe-S) component, which contains 60 and 58 kDa subunits [1]. The fifth subunit of the complex (71 kDa) has not been characterized. The Ni/Fe-S component cleaves the C-C and C-S bond of acetyl-CoA at the

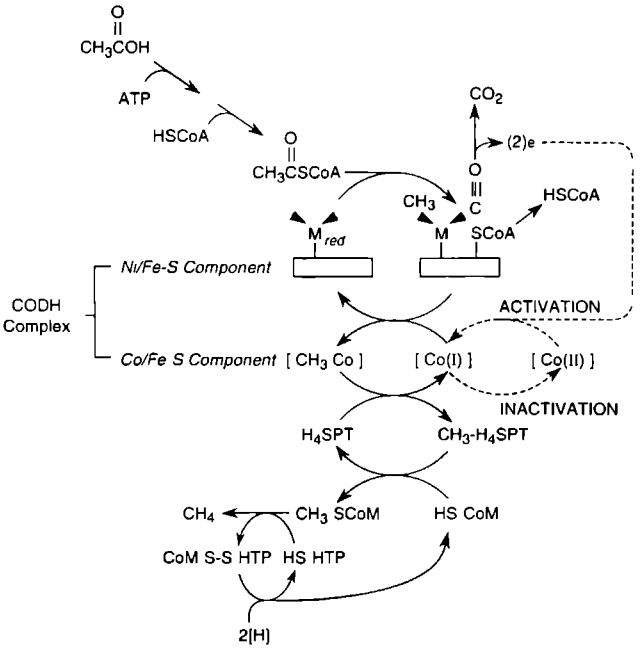

Fig. 6. Scheme of methanogenesis from acetate in *Methanosarcina thermophila* as proposed by Ferry [22] The M above the rectangle represents the active site metal center of the Ni/Fe-S component The cycle of activation-inactivation of the Co/Fe-S component is indicated by the dashed lines [Co(II)] and [Co(I)] represent the oxidation states of the cobalt of the corrinoid prosthetic group of the Co/Fe-S component CODH, carbon monoxide dehydrogenase

Ni-Fe site. Hereafter, the methyl group can be transferred to the corrinoid of the Co/Fe-S component. Electron paramagnetic resonance (EPR) spectroscopy demonstrated that the Co/Fe-S component maintained the 5-hydroxybenzimidazolyl base of the cob(II)amide uncoordinated to the central cobalt atom [32]. Activation requires a reduction to the Co(I) state with a midpoint redox potential ($E^{0'}$) of -486 mV (pH 7.8) and is accomplished by electrons donated directly by the CO-oxidizing Ni/Fe-S component [1,32]. In *M. barkeri* an $E^{0'}$ of -426 mV has been reported for this reduction [29]. Since the CO/CO_2 couple has an $E^{0'}$ of -517 mV, reduction of the cobalt center of the Co/Fe-S component would be exergonic. Indeed, ATP is not required for reduction of the cobalt to the Co(I) state.

The Ni/Fe-S component is also able to reduce the 4Fe-4S cluster of the Co/Fe-S component ($E^{0'}$= -502 mV at pH 7.8) [1,32]. The role of

the Fe-S cluster is unknown but it is suggested that it may be involved in electron transfer from the Ni/Fe-S component to the corrinoid [32]. With the cobalt in the fully reduced Co(I) state the Co/Fe-S component is able to accept the methyl group from the Ni/Fe-S component and a methylated corrinoid intermediate is produced [1,80]. The methyl group is subsequently transferred to H_4SPT [24,28]. The C_1-unit of methyl-H_4SPT is converted to CH_3-S-CoM and methane as described in section 1.3.3. and 1.3.1., respectively.

1.3.5. Methanogenesis from methylamines and methylsulfides

At the biochemical level, methane formation from mono-, di-, and trimethylamine is only poorly investigated. When grown on trimethylamine *M. barkeri* contains a trimethylamine:HS-CoM methyltransferase activity [47]. By the action of this enzyme(system) dimethylamine and CH_3-S-CoM are produced. The reaction requires the presence of ATP and H_2, which suggests a reaction mechanism analogously to the MT_1/MT_2 system (section 1.3.1.). The second methyltransferase could be MT_2 isoenzyme II [87]. Besides dimethylamine, monomethylamine is also observed as a transient intermediate during the conversion of trimethylamine by cell extracts [47]. This and the fact that methanol-cultured cells are unable to convert mono-, di-, or trimethylamine could suggest that these three substrates are each converted by distinct inducible enzymes.

A selected number of methylotrophic methanogens are capable of growth on dimethylsulfide. Methanethiol is transiently produced during growth on dimethylsulfide but can also be used as a substrate for growth [48]. Cell extracts of methanol- and trimethylamine-cultured cells are unable to convert dimethylsulfide or methanethiol, which suggests that these substrates are converted by distinct inducible enzymes [48].

1.4. Methyltransferase reactions in non-methanogens

Methyl group transfers are associated with a number of key cellular functions, including regulation of gene expression, protein synthesis, and metabolic processes such as neurotransmitter biosynthesis. While the bulk of biological methyl transfer reactions use S-adenosyl-methionine (AdoMet), a condensation product of ATP and methionine, as the methyl donor [33], some reactions employ methyl-corrinoids.

Besides their function in methanogenesis (see above), corrinoid-dependent methyltransferases are also involved in biosynthesis of methionine and acetate [44]. The methyl transfer reactions concerned with the production of the latter two compounds will be discussed briefly.

1.4.1. Methionine synthesis

Cobalamin-dependent methionine synthase (EC 2.1.2.13) catalyzes the transfer of the methyl group from 5-methyl-tetrahydrofolate to homocysteine, to produce tetrahydrofolate and methionine. During turnover, the cobalamin cofactor of methionine synthase shuttles between methylcobalamin and cob(I)alamin [4]. As isolated from *Escherichia coli*, the 136-kDa protein is inactive, with cobalt in the Co(II) form. Activation requires catalytic amounts of the highly reactive methyl donor AdoMet and a reducing system with a redox range of about -350 mV [3]. The inactive Co(II) state of the enzyme has an $E^{0'}$ as high as -526 mV for the Co(II)-to-Co(I) reduction [3]. Here, AdoMet is the driving force for the thermodynamically unfavorable reduction of cob(II)alamin: it facilitates the reduction by trapping traces of cob(I)alamin formed as methylcobalamin [3]. It should be noted that neither AdoMet nor its methanogenic structural counterpart S-adenosylmethylcoenzyme M are involved in the reductive activation of corrinoid-dependent methanogenic methyltransferases [78].

The crystal structure of a 27-kDa methylcobalamin-binding fragment of methionine synthase revealed that the 5,6-dimethylbenzimidazole ligand of the corrinoid is not coordinated to the cobalt, but is kept instead in an hydrophobic pocket [19,20]. The lower ligand position is substituted by histidine 759 of the protein. It is suggested that the protein is able to modulate the reactivity of the corrinoid prosthetic group in this way [19].

1.4.2. Acetate synthesis

The acetogenic bacteria produce acetate from two more or less reduced C_1-compounds via the energy-yielding reductive acetyl-CoA pathway [17]. The central enzyme in this pathway is CODH, which synthesizes acetyl-CoA from a methyl group donated by a Co/Fe-S protein, a carbonyl group (CO), and HS-CoA. In fact, the reaction is identical but oppositely directed to the cleavage of acetyl-CoA during

methanogenesis from acetate (section 1.3.4.). The methyl group bound to the cobamide of the Co/Fe-S protein is derived from methyl-tetrahydrofolate. Synthesis of acetate has been most thoroughly studied in *Clostridium thermoaceticum* [52]. Here, the 88-kDa Co/Fe-S protein is isolated with the cob(II)amide in an inactive "base-off" form [52]. Activation requires a reduction to the Co(I) state with an $E^{0'}$ of -504 mV [31]. Electrons derived from the oxidation of CO to CO_2 catalyzed by CODH ($E^{0'}$ = -517 mV) can accomplish this reduction [52]. ATP is not required for the activation process.

In the acetogen *Sporomusa ovata* corrinoid-dependent methyltransferases are also involved in synthesis of methyl-tetrahydrofolate from methanol and methoxybenzoates. Methyl transfer from methanol to tetrahydrofolate is catalyzed by a 40-kDa corrinoid-containing protein [60,64]. Conversion of 3,4-dimethoxybenzoate requires a different methyltransferase. Both reactions are strictly dependent on Ti(III)citrate and catalytic amounts of ATP, that may be required for the reduction of the cobalt of the corrinoid cofactors to the Co(I) form [64]. The corrinoid of the 40-kDa methyltransferase has a *p*-cresolyl-group as its lower ligand, which cannot coordinate to cobalt [61]. Instead, a histidine ligand from the protein ligates to the cobalt atom [60,61]. It has been suggested that this lends the cob(I)amide a remarkable stability towards oxidation [60,61].

Although the stimulating effect of ATP on methanogenesis from methanol was already reported in 1963, the exact mechanism by which this universal energy carrier acts upon that process is still unknown. To elucidate this mechanism the strictly ATP-dependent methanol:HS-CoM methyltransferase reaction was studied in *M. barkeri* strain MS.

In Chapter 2 the enhanced resolution of the components involved in the methanol:HS-CoM methyltransferase reaction is reported. Here, it is revealed that besides MT_1, MT_2, hydrogenase, and ferredoxin, one more protein is required for the overall reaction and more specifically in the activation of MT_1. The component is called Methyltransferase Activation Protein (MAP).

The purification and characterization of the ferredoxin concerned with the activation of MT_1 is described in Chapter 3. The redox properties of the 2[4Fe-4S] cluster of the protein are extensively studied.

To figure out what makes B_{12}-HBI the preferred candidate as a prosthetic group for methanogenic corrinoid proteins as opposed to the ubiquitously occurring cobalamin (B_{12}-DMBI), the electrochemistry of the former corrinoid will be studied in Chapter 4. It is demonstrated here that the HBI base is the weaker ligand, and thus favors "base-off" formation as compared to the DMBI base of cobalamin.

In Chapter 5 the purification of MAP is reported. The protein was shown to interact with ATP during the activation of MT_1. Moreover, MAP became phosphorylated by catalyzing the transfer of the γ-phosphoryl group of ATP, which elucidated the function of ATP during the activation of MT_1.

Chapter 6 describes the activation mechanism of MT_1. Here, the function of MAP and ATP is discussed in relation to the reduction of the corrinoids of MT_1 to the Co(I) level.

In addition, the first step in the oxidation of methanol was studied (Chapter 7). The methyl group of methanol was shown to be directly transferred to H_4MPT. The reaction was catalyzed by a membrane-associated protein.

Finally, in Chapter 8 results with respect to the conversion of methanol by *M. barkeri* and the reductive activation of corrinoid containing methyltransferases in methanogens are summarized as conclusively remarks.

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Involvement of an activation protein in the methanol: 2-mercaptoethanesulfonic acid methyltransferase reaction in *Methanosarcina barkeri*

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Involvement of an Activation Protein in the Methanol:2-Mercaptoethanesulfonic Acid Methyltransferase Reaction in *Methanosarcina barkeri*

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Methanol:5-hydroxybenzimidazolylcobamide methyltransferase (MT_1) is the first of two enzymes required for transfer of the methyl group of methanol to 2-mercaptoethanesulfonic acid in *Methanosarcina barkeri*. MT_1 binds the methyl group of methanol to its corrinoid prosthetic group only when the central cobalt atom of the corrinoid is present in the highly reduced Co(I) state. However, upon manipulation of MT_1 and even during catalysis, the enzyme becomes inactivated as the result of Co(I) oxidation. Reactivation requires H_2, hydrogenase, and ATP. Ferredoxin stimulated the apparent reaction rate of methyl group transfer. Here we report that one more protein fraction was found essential for the overall reaction and, more specifically, for formation of the methylated MT_1 intermediate. The more of the protein that was present, the shorter the delay of the start of methyl group transfer. The maximum velocity of methyl transfer was not substantially affected by these varying amounts of protein. This demonstrated that the protein was involved in the activation of MT_1. Therefore, it was called methyltransferase activation protein.

Methanosarcina barkeri is a methanogenic bacterium which can grow on various one-carbon compounds such as CO_2, methylamines, and methanol and on acetate (5). Growth on methanol can occur in the absence and presence of H_2, in the latter case, the growth medium must be supplemented with acetate to ensure growth (9).

The reduction of methanol to methane occurs via methyl-coenzyme M (CH_3-S-CoM), the substrate for the final step in methanogenesis in all methanogens studied so far (5). Synthesis of CH_3-S-CoM from methanol and coenzyme M (2-mercaptoethanesulfonic acid; HS-CoM) is catalyzed by the concerted action of two methyltransferases. First, methanol:5-hydroxybenzimidazolylcobamide (B_{12}-HBI) methyltransferase (MT_1) binds the methyl group of the substrate to its corrinoid prosthetic group (14). Next, the methyl group is transferred to HS-CoM by Co-methyl-5-hydroxybenzimidazolyl-cobamide:HS-CoM methyltransferase (MT_2) (12).

MT_1 is catalytically active only when the central cobalt atom of its corrinoid prosthetic group is present in the highly reduced Co(I) state (B_{12}-HBI_s) (15). However, upon manipulation of MT_1 and even during catalysis in a resolved system, the enzyme becomes inactivated as the result of B_{12}-HBI_s oxidation. Reactivation is possible and requires a reducing system (H_2, hydrogenase, ferredoxin) and ATP (13, 15).

Here, evidence is presented that the methyl group transfer of methanol to HS-CoM is dependent on one more protein fraction. The function of the protein is discussed in relation to the reductive activation of MT_1 and the name methyltransferase activation protein (MAP) is suggested.

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MATERIALS AND METHODS

Culture methods and preparation of cell extract. Cells of *M. barkeri* MS (DSM 800) were cultured in a 300-liter fermentor in a mineral medium with methanol as a substrate as described before (4). Cells were harvested anaerobically at the end of the exponential phase and stored at −70°C under N_2.

Cell extract was prepared by suspension of wet cells (1:1 [wt/vol]) in 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer (pH 7.0) containing 15 mM $MgCl_2$, 1 mM dithiothreitol, RNase (10 μg ml^{-1}), and DNase (10 μg ml^{-1}) followed by passage through a French pressure cell at 138 MPa under continuous flushing with N_2. Centrifugation at 13,200 × *g* (20 min, 4°C) pelleted cell debris and unbroken cells. The supernatant, referred to as cell extract, was collected and stored at −70°C under H_2.

Enzyme assays. Incubation mixtures were prepared in an anaerobic glove box, and the reaction was performed in crimp-sealed 10-ml serum vials. Unless stated otherwise, a typical reaction mixture (final volume, 200 μl) contained 100 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer (pH 7.0), 24 mM $MgCl_2$, 10 mM methanol, 10 mM HS-CoM, 1 mM ATP, 1 mM 2-bromoethanesulfonic acid (to prevent possible enzymic reduction of CH_3-S-CoM to methane), 40 μl of MT_2/hydrogenase fraction (0.83 mg of protein ml^{-1}; 1.12 μmol of HS-CoM converted min^{-1} mg of $protein^{-1}$; 0.14 μmol of benzylviologen reduced min^{-1} mg of $protein^{-1}$; 0.03 μmol of coenzyme F_{420} reduced min^{-1} mg of $protein^{-1}$), 25 μl of MT_1 fraction (1.15 mg of protein ml^{-1}; 0.62 μmol of HS-CoM converted min^{-1} mg of $protein^{-1}$), 10 μl of ferredoxin fraction (0.12 mg of protein ml^{-1}; 0.45 μmol of dithiodiethanesulfonic acid reduced min^{-1} mg of $protein^{-1}$), and 50 μl of MAP fraction (1.88 mg of protein ml^{-1}). After gassing with 50% H_2–50% N_2 (100 kPa), the vials were kept on ice. Reactions were started by placing the vials at 37°C. After appropriate incubation periods, generally 0, 20, 40, and 60 min, reactions were stopped by placing the vials on ice. Activity of methyl group transfer of methanol to

HS CoM was routinely assayed by measuring the decrease in the amount of HS CoM

The enzymatic activities of MT_1 and MT_2 were determined by measuring the rate of HS CoM decrease as described above with 40 μl of concentrated MT_1 or MT_2 fraction or 130 μl of column fraction together with 8 μl of cell extract Here, cell extract substituted for the other enzymic components Methyltransferase activity of 8 μl of cell extract alone was almost negligible MT_2 activity was qualitatively determined by following the formation of B_{12} (brown) from methylcobalamin (red) and HS CoM, as described by Kengen et al (6) Reaction mixtures contained 2 5 mM methylcobalamin, 5 mM HS CoM, and 100 μl of protein fraction, 100 mM *N* tris(hydroxymethyl)methyl 2 amino ethanesulfonic acid buffer (pH 7 0) was added to a final volume of 200 μl Incubation was for 30 min at room temperature in the anaerobic glove box

Hydrogenase activity was tested as described by Fiebig and Friedrich (2) with benzylviologen or coenzyme F_{420} as a substrate Benzylviologen reduction was monitored at 578 nm (ϵ_{578} = 8 6 mM $^{-1}$ cm $^{-1}$) Reduction of coenzyme F_{420} was monitored at 401 nm (ϵ_{401} = 26 0 mM $^{-1}$ cm $^{-1}$)

Ferredoxin dependent activity was determined by measuring the ferredoxin dependent reduction of dithiodiethane sulfonic acid to its monomers in the presence of H hydrogenase, and aquocobalamin as described before (15) MT_2/hydrogenase fraction was used as a source of hydrogenase

MAP activity of column fractions was tested as described for the overall methyltransferase assay, except that 70 μl of fraction was used as a source of MAP

Fractionation of cell extract. Several enzymes involved in transfer of the methyl group of methanol to HS CoM are oxygen labile (14, 15) Therefore, all handlings were performed in an anaerobic glove box (97 5% N_2–2 5% H_2) Cell extract (10 ml, 260 mg of protein) was centrifuged for 15 min at 16,000 × *g* (Eppendorf) The supernatant was applied to a DEAE Sepharose Cl 6B column (12 by 2 8 cm) equilibrated with 50 mM *N* tris(hydroxymethyl)methyl 2 aminoethane sulfonic acid buffer (pH 7 0) containing 15 mM $MgCl_2$ and 1 mM dithiothreitol (buffer A) Elution with 100 ml of buffer A yielded a pass through fraction Bound proteins and cofactors were eluted with a 400 ml linear gradient of 0 to 0 6 M NH_4Cl in buffer A Finally the column was washed with 50 ml of 0 6 M NH_4Cl in buffer A The eluate was monitored at 280 nm, and 5 ml fractions were collected at a flow rate of 1 ml min $^{-1}$

Fractions eluting between 0 20 and 0 22 M NH_4Cl yielded the MT_2 fraction which also contained hydrogenase activity Fractions eluting between 0 25 and 0 34 M NH_4Cl contained MAP activity MT_1 was present in fractions eluting between 0 39 and 0 42 M NH_4Cl Ferredoxin was obtained between 0 50 and 0 56 M NH_4Cl Since NH_4Cl is an inhibitor of the methanol conversion in a resolved system (14), MT_2/hydrogenase, MT_1, and ferredoxin fractions were washed by ultrafiltration (Amicon YM 10, PM 30, and YM 3 filters, respectively) with buffer A and concentrated to a final volume of 3 ml Fractions containing MAP were washed by ultrafiltration (Amicon PM 30 filter) with buffer A and concentrated to a final volume of 6 ml Ethylene glycol was added as a stabilizing agent to the MT_1, MAP and ferredoxin fractions in concentrations of 10 10 and 20% (vol/vol), respectively

Extraction of corrinoids. Reaction mixtures for corrinoid analysis were essentially the same as described for the overall methyltransferase assay, except that no HS-CoM was added and the volume of the reaction mixtures was increased to 400 μl Because of the light sensitivity of methylated corrinoids, the vials were wrapped in aluminum foil and all handlings were carried out in the dim light of a red lamp Incubation was for 60 min at 37°C under H_2 atmosphere After incubation, 4 volumes of 96% ethanol were added, and corrinoids were extracted by heating the reaction mixture for 30 min at 90°C Denatured proteins were pelleted by centrifugation (Eppendorf, 15 min, 16,000 × *g*) and resuspended in an equal volume of 80% ethanol, whereupon the extraction and centrifugation were repeated The supernatants obtained were combined and dried in vacuo at 50°C The residue was dissolved in 300 μl of distilled water, centrifuged (Eppendorf, 10 min, 16,000 × *g*), and filtered over a 0 45 μm HV luer lock filter (Millipore, Bedford, Mass) Aliquots of 200 μl were subjected to reversed phase high performance liquid chromatography (HPLC) analysis

When ^{14}C methylated corrinoids were extracted, reaction mixtures were the same as described above, except that 10 mM [^{14}C]methanol (0 13 TBq mol $^{-1}$) was used Incubation and extraction were performed as described above, except that the residue obtained was dissolved in 100 μl of 80% ethanol Aliquots of 30 μl were spotted on thin layer chromatography plates, developed, and analyzed

Analytical methods. The amount of HS CoM was determined by the method of Ellman (1) Samples of 25 μl were mixed with 3 ml of 0 48 mM 2 2 -dinitro 5 5 dithiobenzoic acid in 150 mM Tris Cl buffer (pH 8 0) and measured immediately at 412 nm The methanol concentration was determined by gas chromatography as described before (13) except that a final concentration of 3 mM 2 propanol was used as an internal standard The protein concentration was determined with the Coomassie brilliant blue G 250 method (10) and bovine serum albumin as a standard HPLC analysis was performed at 35°C on a Hewlett-Packard 1048B HPLC, equipped with a Hewlett-Packard 1040A diode array detector and a 5 μm LiChrosorb RP 18 column (150 by 46 mm, Alltech Europe, Eke, Belgium) A linear gradient of 8 to 72% methanol in 25 mM sodium acetate buffer (pH 6 0) was applied to the column in 25 min at a flow rate of 0 8 ml min $^{-1}$ The eluate was monitored at four pilot wavelengths 260, 361, 518 and 550 nm When a peak was recognized as such, complete UV visible light spectra (210 to 600 nm) were recorded continuously Peaks were identified by their retention times and UV visible light spectra The retention times of aquo B_{12} HBI and methyl B_{12} HBI were 13 1 and 17 5 min, respectively Thin layer chromatography was performed on silica gel plates from Merck (DC Plastikfolien Kieselgel 60 0 2 mm) developed with methanol acetic acid-water (7/1/12 [vol/vol]) The R_f values of aquo B_{12} HBI and methyl B_{12} HBI were 0 32 and 0 47, respectively Radioactivity was located by autoradiography for 4 days with Kodak XAR 5 X ray film and was quantitatively determined by cutting the thin layer chromatography plates, scratching off the silica gel, and dissolving the labeled compound in 1 ml of water and then in 10 ml of Lumagel scintillation fluid (Lumac, Schaesberg, The Netherlands)

Materials. HS CoM, 2 bromoethanesulfonic acid, *N* tris (hydroxymethyl)methyl 2 aminoethanesulfonic acid, and methylcobalamin were purchased from Sigma Chemical Co (St Louis Mo) Dithiothreitol was from Serva Feinbiochemica (Heidelberg, Germany) ATP was purchased from Boehringer (Mannheim, Germany) DEAE Sepharose Cl 6B was from Pharmacia LKB Biotechnology A B (Uppsala, Sweden) Methanol (HPLC grade) was obtained from J T Baker (Deventer, The Netherlands) [^{14}C]methanol (0 13 TBq mol $^{-1}$) came from New England Nuclear (Boston,

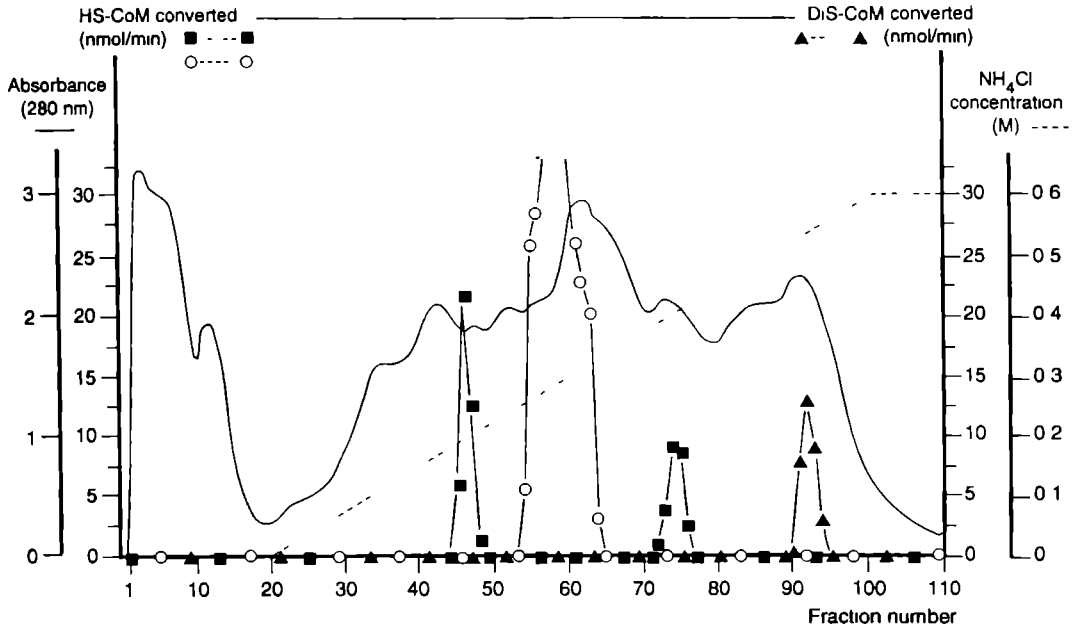

FIG 1 Resolution of the methyltransferase system of *M barkeri* by DEAE Sepharose Cl 6B ion exchange chromatography Elution of cell extract (260 mg of protein) and enzyme assays were performed as described in Materials and Methods Incubations were under 50% H_2-50% N_2 for 60 min at 37°C When the rate of HS-CoM decrease was determined in the presence of 8 μl of cell extract and 130 μl of fraction, two components which stimulated in the overall assay were found (-■) The activity eluting between 0 20 and 0 22 M NH_4Cl was caused by MT_2, which also contained hydrogenase activity The activity eluting between 0 39 and 0 42 M NH_4Cl was caused by MT_1 Ferredoxin eluted between 0 50 and 0 56 M NH_4Cl (-▲) Determination of HS CoM conversion in the presence of the concentrated MT_2/hydrogenase, MT_1, and ferredoxin fractions and 70 μl of column fraction revealed MAP activity eluting between 0 25 and 0 34 M NH_4Cl (-○) DiS=CoM, dithioethanesulfonic acid

Mass). Gasses were supplied by Hoek Loos (Schiedam, The Netherlands) To remove traces of oxygen, H_2 containing gasses were passed over a BASF RO-20 catalyst at room temperature and nitrogen was passed over a prereduced BASF R3-11 catalyst at 150°C The catalysts were a gift of BASF Aktiengesellschaft (Ludwigshafen, Germany)

RESULTS

Reconstitution of the methyltransferase system. After fractionation of cell extract of *M barkeri* on DEAE-Sepharose, MT_2, MT_1, and ferredoxin were completely separated (Fig 1) The MT_2 fraction also contained hydrogenase activity capable of reducing both coenzyme F_{420} and benzylviologen Therefore, this fraction was also used as a source of hydrogenase and called MT_2/hydrogenase fraction When methanol conversion was determined in the presence of the MT_2/hydrogenase, MT_1, and ferredoxin fractions, no decrease in HS-CoM concentration was detected (Fig 2) This indicated that a component involved in methyl transfer of methanol to HS-CoM was missing Indeed, methanol conversion in the presence of the above mentioned components required an additional fraction that was eluted between 0 25 and 0 34 M NH_4Cl, notably between MT_2/hydrogenase and MT_1 (Fig 1) Addition of this fraction, after washing and concentration on a PM-30 ultrafiltration membrane, restored CH_3-S CoM synthesis from methanol in the presence of MT_2/hydrogenase, MT_1, and ferredoxin (Fig 2) The same results were obtained when the reactions were monitored by measuring the decrease in methanol (data not shown) The methanol decrease paralleled the decrease in HS-CoM, which was in agreement with a 1 1 stoichiometry of methanol and HS-CoM conversion as already established by Shapiro and Wolfe (11) In the absence of methanol, no detectable HS CoM conversion occurred

The finding that activity of the fraction eluting between 0 25 and 0 34 M NH_4Cl was retained by a PM-30 filter (nominal molecular weight cutoff of 30,000) indicated that the activity was derived from a protein(s) In agreement with this, activity was destroyed by heating at 100°C for 5 min In view of the results described below, the pertinent protein will be designated methyltransferase activation protein (MAP) It should be noted that the MAP fraction obtained after DEAE Sepharose separation was not pure and that polyacrylamide gel electrophoresis revealed the presence of several protein bands (data not shown)

MAP fraction alone was not capable of methanol-dependent HS CoM conversion to CH_3-S-CoM (Fig 2) Neither did the reaction proceed in the absence of either MT_2/hydrogenase or MT_1 (Fig 2) Ferredoxin was not strictly required, though its presence stimulated the initial reaction rate about twofold (Fig 2)

Role of MAP and ATP in the methyltransferase reaction. When methyl group transfer of methanol to HS CoM in the

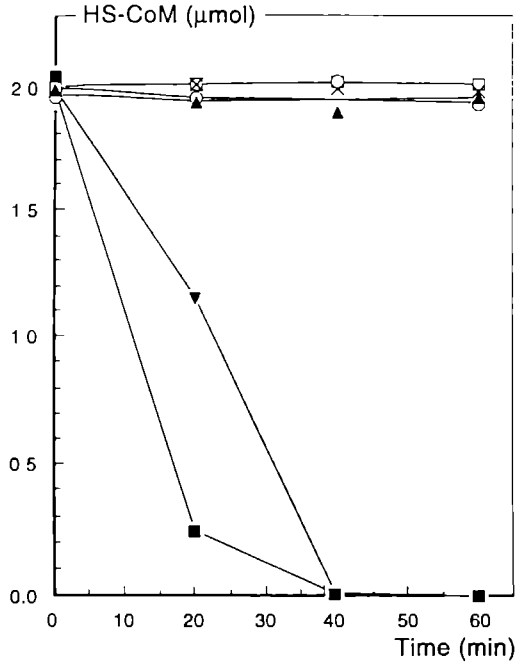

FIG 2 Requirement for various proteins in the methyl group transfer of methanol to HS-CoM Reaction mixtures (final volume, 200 μl) contained 100 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer (pH 7 0), 24 mM $MgCl_2$, 10 mM methanol, 10 mM HS CoM, 1 mM ATP, 1 mM 2-bromoethanesulfonic acid, 40 μl of MT_2/hydrogenase fraction, 25 μl of MT_1 fraction, 10 μl of ferredoxin fraction, and 50 μl of MAP fraction Incubations were performed under 50% H_2–50% N_2 at 37°C. Results from the complete assay (■) or assays omitting MAP (-×-), MT_2/hydrogenase (-○-), MT_1 (-▲-), or ferredoxin (-▼-) are shown The control experiment (-□-) contained no protein fractions other than MAP.

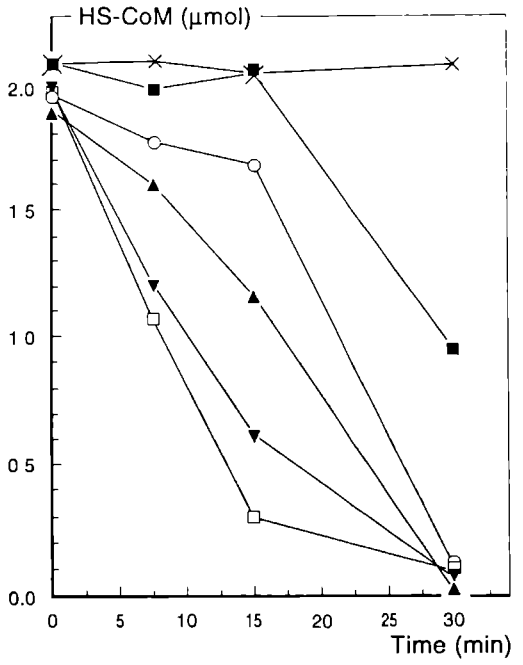

FIG 3. Effect of various amounts of MAP on methyl group transfer of methanol to HS-CoM Reactions were performed as described for Fig. 2, except that the amount of MAP was varied, none (-×-), 5 μl (-■-), 10 μl (-○-), 15 μl (-▲-), 25 μl (-▼-), or 50 μl (-□-)

presence of MT_2/hydrogenase, MT_1, and ferredoxin was monitored with various amounts of MAP, it appeared that the latter primarily affected the onset of the reaction (Fig. 3). Increasing amounts of MAP resulted in a decrease of the lag phase. Once the reaction had started, maximum velocities were not substantially affected by the varied amounts of MAP applied. When MAP fraction was present in sufficient amounts (50 μl), the reaction started immediately. Exposure of MAP to air for increasing periods of time and subsequent removal of air resulted in the increase of the lag phase of the methyltransferase reaction. No activity was recovered after a 24-h air exposure of MAP (data not shown). This indicated that MAP is an oxygen-labile protein.

Methyl group transfer of methanol to HS-CoM is strictly dependent on ATP (13). Addition of various amounts of ATP had a dual effect on the methyltransferase reaction (Fig. 4). Increasing amounts of ATP led to a decrease of the lag phase and an increase in the reaction rate. With 200 nmol of ATP, the reaction rate was maximal and a lag phase was absent. However, essentially the same results were obtained with a 10-fold-lower amount of ATP Since 2 μmol of HS-CoM had been converted at the end of the reaction, ATP is clearly required only in catalytic amounts and one molecule of ATP is sufficient to drive 10 to 100 catalytic cycles. The latter value may even be underestimated, since the MT_2/hydrogenase fraction contained some ATP-hydrolyzing activity, which may have nonspecifically consumed part of the ATP.

CH_3-S-CoM synthesis from methanol proceeds by binding of the methyl group to the corrinoid prosthetic group of MT_1, followed by the MT_2-catalyzed methyl group transfer to HS-CoM (12). In order to study the effect of MAP and ATP on the first reaction, methanol was incubated with various protein fractions and ATP in the absence of HS-CoM. Corrinoid analysis by HPLC showed that B_{12}-HBI was almost exclusively extracted as its methyl derivative (Fig. 5A). No methyl-B_{12}-HBI was formed when either ATP or MAP was omitted (Fig. 5B and C). Remarkably, methyl-B_{12}-HBI was also produced when methanol was not further added (Fig. 5D). However, buffers and protein solutions always contained low concentrations (maximally about 50 μM) of this compound (16) originating from methanol vapor in the glove box atmosphere. (The solvent is routinely used in high concentrations for the anaerobic purification of oxygen-labile methanogenic coenzymes.) In order to verify that methanol was indeed the source of the methyl group of methyl-B_{12}-HBI, we repeated the experiments with [^{14}C] methanol (0.53 MBq). Autoradiography of the corrinoids extracted revealed that label was incorporated when MAP, ATP, and [^{14}C]methanol were present. The radioactive spot had the same R_f value as authentic methyl-B_{12}-HBI (0.47) and coincided with a pink color (data not shown) The

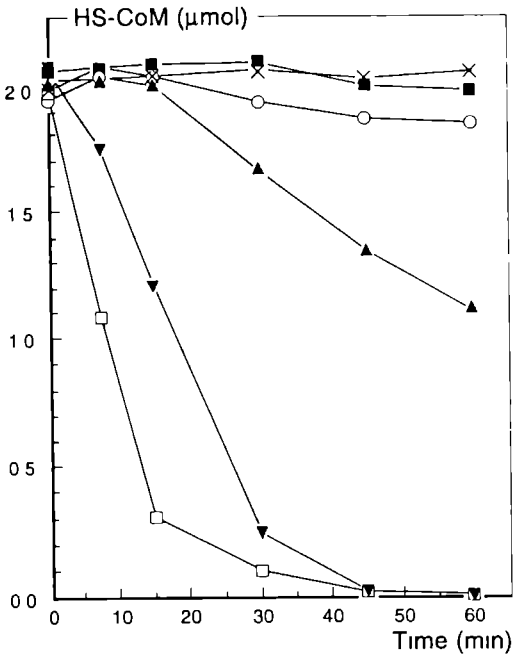

FIG 4 Effect of various amounts of ATP on methyl group transfer of methanol to HS CoM Reactions were performed as described for Fig 2, except that the amount of ATP was varied, none (-×-), 2 nmol (-■-), 5 nmol (-○-) 10 nmol (-▲-) 20 nmol (-▼-) or 200 nmol (-⊟-)

amount of label incorporated was 4,193 Bq In the absence of MAP, ATP, or [^{14}C]methanol, no spots were observed on the autoradiogram Quantitative determination of the amount of radioactivity around the R_f 0 47 zone on the chromatogram in the absence of MAP, ATP, or [^{14}C]methanol yielded only 6, 2, and 1 Bq, respectively

DISCUSSION

In *M barkeri*, CH_3-S-CoM synthesis from methanol and HS-CoM proceeds by the participation of MT_1, which contains the corrinoid B_{12} HBI as the catalytic center, and MT_2 (12, 14) (Fig 6) With the central cobalt atom in the highly reduced Co(I) state, B_{12} HBI_s acts as a powerful nucleophile capable of withdrawing the methyl group from methanol, and thus methyl-B_{12} HBI is formed B_{12} HBI_s, however, is extremely sensitive towards oxidation Methyl-B_{12} HBI is an oxygen stable compound, but here the Co C bond is readily cleaved by the action of light leaving aquo B_{12} HBI, with cobalt in the Co(III) state as the photolysis product As the result of B_{12} HBI_s oxidation and methyl B_{12}-HBI photolysis, MT_1 is isolated in an inactive state From the work of van der Meijden et al (12–15), it is known that *M barkeri* contains a reactivation system In agreement with the results of those authors, we found that reactivation required the presence of a reducing system composed of H_2, hydrogenase, and ferredoxin, as well as catalytic amounts of ATP In our study, ferredoxin was dispensable, though it stimulated

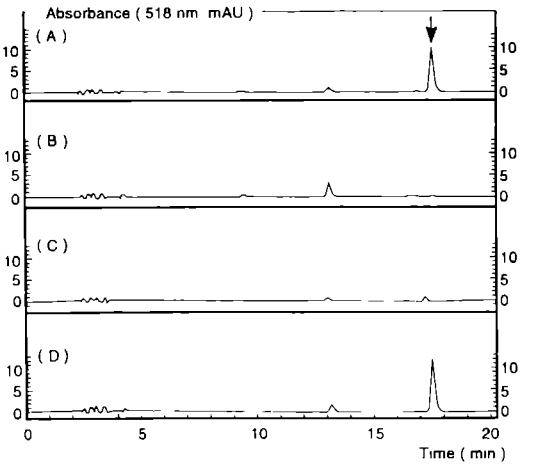

FIG 5 HPLC analysis of corrinoids Incubation and corrinoid extraction took place as described in Materials and Methods (A) Complete reaction mixture (24 mM $MgCl_2$ 10 mM methanol, 1 mM ATP, 1 mM 2 bromoethanesulfonic acid, 80 µl of MT_2/hydrogenase, 50 µl of MT_1, 20 µl of ferredoxin, and 100 µl of MAP fraction), (B) no ATP added, (C) no MAP added, (D) no methanol added The peak at 17 5 min is methyl B_{12} HBI (arrow) The peak at 13 1 min was identified as aquo B_{12} HBI mAU, milli absorbance units

the apparent reaction rate of methyl group transfer Using the better resolution properties of DEAE Sepharose-Cl-6B, we found that one more oxygen-sensitive protein, termed MAP, was needed for methyl group transfer of methanol to HS CoM In fact, van der Meijden et al (14) may also have encountered the protein in their studies They found that the

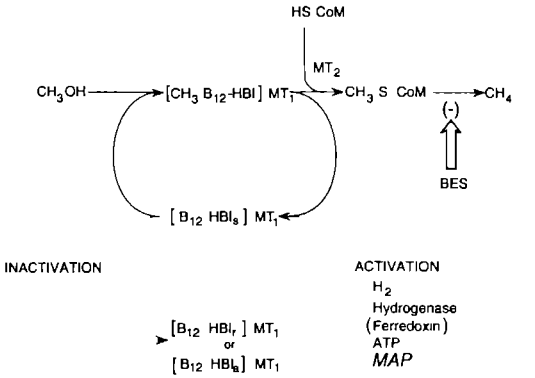

FIG 6 Scheme of transfer of the methyl group from methanol to HS CoM and the position of MAP in that reaction [B_{12} HBI_a], [B_{12} HBI_r], and [B_{12} HBI_s] represent the Co(III), Co(II), and Co(I) states of reduction, respectively, of the corrinoid prosthetic group of MT_1 BES, 2 bromoethanesulfonic acid Ferredoxin is shown in parentheses because methyl group transfer can occur in the absence of this protein, though it stimulates the apparent reaction rate of methyl group transfer The cycle of activation inactivation of MT_1 is indicated by the dashed lines

activity of MT_1 was completely lost in the final purification step. Activity, however, was (partly) recovered by addition of a protein fraction, named component S, that became separated from MT_1 during the purification step. However, component S is too poorly defined to allow a proper comparison with MAP.

When present in increasing amounts, MAP caused the decrease of the lag phase in the onset of the reaction. Moreover, MAP was necessary for the formation of methyl-B_{12}-HBI. These results suggest that MAP is involved in some initial event in the process, the conversion of inactive MT_1 into a catalytic-competent enzyme. Activation of MT_1 must imply a reduction of Co(III) and Co(II) to Co(I). In free corrinoids, the Co(II)-to Co(I) reduction shows a midpoint potential as low as −640 mV (7). With H_2 (−414 mV) as reductant, the equilibrium level of B_{12}-HBI_s is extremely low and MT_1 apparently remains inactive. With respect to the role of MAP and of ATP, two modes of action may then be envisaged: (i) Coupled to ATP hydrolysis, the reduction potential of electrons derived from H_2 oxidation is driven to a level which permits Co(II)-to Co(I) reduction. Here, the role of ATP and MAP might resemble the one in the nitrogenase reaction (3, 8). (ii) By the action of ATP and MAP, MT_1 or its corrinoid prosthetic group is modified in such a way that reduction of Co(II) to Co(I) by hydrogenase becomes feasible. In both mechanisms, B_{12}-HBI_s may be trapped as methyl-B_{12}-HBI by methanol present in the assay. Future investigations with the purified MAP protein must decide which mechanism will hold.

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Characterization and determination of the redox properties of the 2[4Fe-4S] ferredoxin from *Methanosarcina barkeri* strain MS

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Characterization and determination of the redox properties of the 2[4Fe-4S] ferredoxin from *Methanosarcina barkeri* strain MS

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Abstract Ferredoxin was purified from methanol grown *Methanosarcina barkeri* strain MS. It was isolated as a dimer with a subunit molecular weight of 6 200. The protein contained 7.4 mol iron and 7.2 mol acid labile sulfur per monomer. In the reduced state the ferredoxin exhibited an EPR spectrum characteristic of two spin coupled [4Fe-4S] clusters. The E_m of the [4Fe-4S] couple was −322 mV ± 3 mV vs. NHE at 21°C and pH 7.0. The midpoint potential was temperature but not pH dependent. At the physiological temperature of 37°C the E_m was −340 mV.

Key words: Ferredoxin; Iron-sulfur cluster; Cyclic voltammetry; Redox potential; *Methanosarcina barkeri*

1 Introduction

Ferredoxins are small acidic proteins involved in the electron transport in a wide variety of redox reactions. The proteins typically contain non heme iron and acid labile sulfur coordinated by cysteines. Ferredoxins have been isolated from many organisms including methanogenic bacteria [1,2].

Ferredoxins have been purified from various *Methanosarcina* species, including *M. barkeri* strain MS [3,4], *M. barkeri* strain Fusaro [5] and *M. thermophila* [6]. The ferredoxin of *M. barkeri* strain MS is composed of 59 amino acids with a molecular mass of 6 000. It has a 41% sequence homology with the 2[4Fe-4S] ferredoxin from *Clostridium pasteurianum* [7]. The eight cysteine residues involved in cluster formation in the *C. pasteurianum* ferredoxin are completely conserved in the ferredoxin from M. barkeri [1,7]. However, as isolated the latter protein was found to contain a 3Fe cluster [3]. The ferredoxin from M. barkeri strain Fusaro has a molecular mass of 6 100. The protein contains 7 iron and 7–8 acid labile sulfur atoms per molecule indicative of the presence of 2[4Fe-4S] clusters [5]. The ferredoxin gene from M. thermophila has been sequenced and encodes a 6 200 molecular weight protein with an eight cysteine spacing typical for a 2[4Fe-4S] protein [8]. Resonance Raman and electronic paramagnetic resonance (EPR) spectroscopy studies demonstrated that the protein indeed contained two [4Fe-4S] clusters per monomer [9].

From the above it is clear that there is a notable difference between the amount of iron and sulfur expected on the basis of the amino acid sequence and the iron-sulfur content present in the as isolated ferredoxin from *M. barkeri* strain MS [3]. In this communication we demonstrate that the ferredoxin from *M. barkeri* strain MS can be purified as a 2[4Fe-4S] protein. Its redox properties were determined.

2. Materials and methods

2.1 Organism

Methanosarcina barkeri strain MS (DSM 800) was grown in a mineral medium with methanol as a substrate. Cell free extract was prepared in 50 mM TES/K buffer (pH 7.0) containing 15 mM MgCl and 1 mM dithiothreitol and stored at −70°C under H [10].

2.2 Purification of ferredoxin

Because of the oxygen sensitivity of ferredoxin, all handlings were performed under anaerobic conditions [10]. Cell free extract (10 ml) was applied to a DEAE Sepharose CL 6B column (12 by 2.8 cm) equilibrated in 50 mM TES/K buffer (pH 7.0) containing 15 mM MgCl and 1 mM dithiothreitol. Bound proteins were eluted with a 400 ml linear gradient of 0 to 0.6 M NH_4Cl as described before [10]. The ferredoxin containing fractions eluting between 0.50 and 0.56 M NH_4Cl were concentrated by Amicon YM-3 ultrafiltration to a final volume of 1 ml. Ethylene glycol was added as a stabilizing agent to a final concentration of 10%. Ferredoxin was further purified by gel filtration on Superose 6 (30 by 1 cm) equilibrated in 50 mM TES/K buffer (pH 7.0) containing 15 mM MgCl, 1 mM dithiothreitol, 150 mM NH_4Cl and 10% ethylene glycol. Ferredoxin fractions that were judged to be pure according to native PAGE were concentrated by Amicon UM-05 ultrafiltration. Ferredoxin was stored at −20°C under H in 10 mM TES/K (pH 7.0) buffer containing 65 mM NH_4Cl and 10% ethylene glycol.

2.3 Spectroscopy

EPR spectroscopy was carried out on a Bruker 200 D spectrometer equipped with cryogenics, peripheral equipment and data acquisition/manipulation facilities as described previously [11]. Ultraviolet visible absorption spectra were measured at room temperature using a Hitachi U-3200 spectrophotometer.

2.4 Electrochemistry

Direct electrochemistry (cyclic voltammetry) of 12 μl amounts of ferredoxin (0.87 mg/ml) was performed under argon atmosphere with the nitric acid treated glassy carbon electrode and the micro apparatus and methodology described before [12]. The response of the reduction potential as a function of the temperature was determined by immersing the electrochemical cell in a thermostatted water bath. For the determination of the pH dependence the protein stock solution was diluted 1:3 into 100 mM buffer mixtures (pH 6–10) of the desired pH.

2.5 Other procedures

Ferredoxin activity was assayed as described by Van der Meijden et al. [4]. Protein concentrations were determined by means of amino acid analysis. Ferredoxin was hydrolyzed in vacuo with 5.7 N HCl at 105°C for 24 h. Amino acids were analyzed after derivatization with 9-fluorenylmethyl chloroformate on a Lichrosorb RP-8 column with L-norleucine as an internal standard [13]. Total iron and acid labile sulfur were determined as described by Fish [14] and Rabinowitz [15], respectively. Native and denaturing SDS PAGE and isoelectric focusing were performed with pre-fabricated minigels using the Pharmacia PhastSystem equipment. The gels were fixed with 20% trichloroacetic acid and stained with Coomassie brilliant blue R-350. The native molecular

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weight was determined by gel filtration on Sephadex G50 (120 by 2.5 cm) equilibrated in 10 mM TES/K (pH 7.0) containing 100 mM NH_4Cl and 1 mM dithiothreitol

3. Results and discussion

3.1 Physical and chemical properties

Ferredoxin was anaerobically purified from *M. barkeri* strain MS by a factor of 116 with a recovery of 16%. The native molecular weight estimated by gel filtration chromatography was 12 000. From SDS PAGE a subunit molecular weight of 6 200 was measured. This suggested that ferredoxin was isolated as a dimer. Determination of the total iron and acid labile sulfur content showed the presence of 7.4 Fe/mol and 7.2 S/mol of monomer, respectively. From isoelectric focusing a pI of 4.6 was obtained.

3.2 Spectral properties

The ultraviolet visible absorption spectrum of the as-isolated oxidized ferredoxin showed absorption maxima at 276 and 384 nm and A_{384}/A_{276} of 0.84. After addition of dithionite the absorption at 384 nm decreased as the result of reduction of the iron sulfur clusters. From the absorption spectrum of the as-isolated oxidized ferredoxin a molar extinction coefficient of $\varepsilon_{384} = 28\,700\ M^{-1}\ cm^{-1}$ per monomer ($\varepsilon_{384} = 3\,900\ M^{-1}\ cm^{-1}$ per iron atom) could be calculated. This type of spectrum and extinction coefficient per iron atom is typical of iron sulfur proteins containing [4Fe-4S] centers [16].

The results described above suggested that the ferredoxin was isolated with two [4Fe-4S] clusters per monomer. Therefore the protein was studied by EPR in two oxidation states. In the oxidized state no signal was observed. After reduction with dithionite a signal with g values at 2.03, 1.99, 1.96, 1.92 and 1.88 appeared (Fig. 1). The complex spectrum had the characteristics of a signal resulting from the spin-spin interaction of two paramagnetic $[4Fe\text{-}4S]^{1+}$ clusters [17,18] and it was nearly identical to the one reported for the *M. thermophila* ferredoxin [9]. Except for the absence of a broad lateral line at $g = 2.12$, the signal was similar to that reported for the fully reduced *C. pasteurianum* ferredoxin [18,19]. The lateral line was also absent in the spectrum of the *M. thermophila* ferredoxin [9] which has a 92% sequence identity to the protein described here [8]. From these results we concluded that the ferredoxin of *M. barkeri* was isolated with 2[4Fe-4S] clusters per subunit.

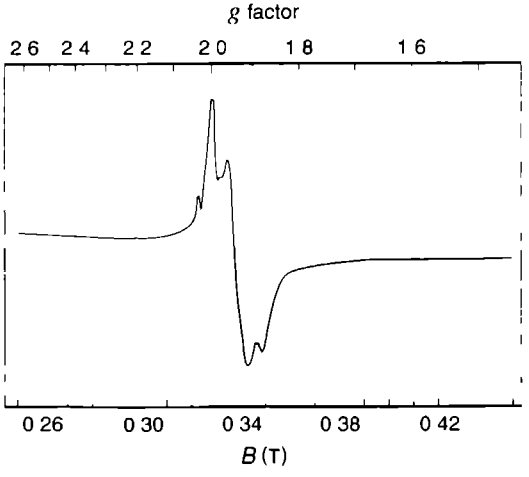

Fig. 1. EPR spectrum of the reduced *M. barkeri* ferredoxin. The protein (0.66 mg/ml) was reduced with 2 mM dithionite and frozen in liquid nitrogen. EPR conditions: microwave frequency 9.1799 GHz; modulation frequency 100 kHz; modulation amplitude 2 mT; microwave power 5.2 mW; temperature 13 K.

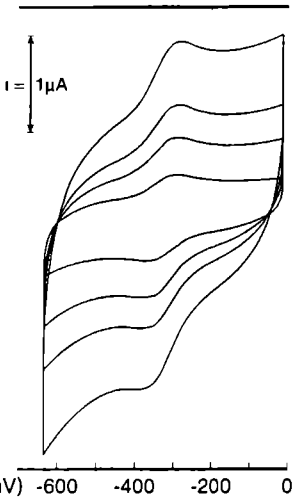

Fig. 2. Cyclic voltammograms of ferredoxin from *M. barkeri*. Conditions: ferredoxin (12 μl, 0.87 mg/ml) in 10 mM TES/K (pH 7.0) containing 65 mM NH_4Cl, 10% ethylene glycol and 3.3 mM neomycin; potential scan rates from the inside outward: 10, 25, 40 and 75 mV/s respectively; temperature 21°C. The potential axis is defined versus the normal hydrogen electrode.

3.3 Reversible electrochemistry

Ferredoxin showed a direct response at the glassy carbon electrode in the absence of promotors or mediators. However in the presence of neomycin (3.3 mM) the signal became more stable and fully reversible. Representative voltammograms are shown in Fig. 2. At low scan rates (typically 10 mV/s) the $n = 1$ electron transfer is reversible, as judged from the reproducible cathodic to anodic peak potential separation of 57 mV ± 5 mV. At ambient temperatures and at neutral pH no significant deterioration of this response was observed over a period of one hour. The reduction potential (E_n) was −322 mV ± 3 mV at 21°C and pH 7.0 versus the normal hydrogen electrode (NHE). From Fig. 2 it is also clear that the midpoint redox potentials of the individual [4Fe-4S] clusters do not differ significantly since only one symmetrical wave is observed.

The response of the reduction potential was also measured as a function of the temperature. At all temperatures measured between 2°C and 50°C the peak-to-peak distance was 58 mV ± 6 mV for a scan rate of 10 mV/s. For temperatures above 50°C reproducibility was not warranted due to desiccation of the sample under the argon flow. At temperatures up to 18°C the midpoint reduction potential decreased linearly with a slope

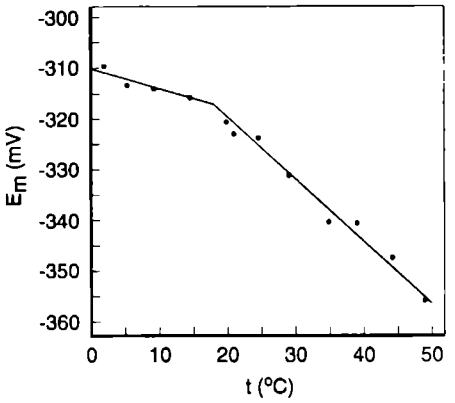

Fig 3 Temperature dependence of the midpoint redox potential of the ferredoxin from *M barkeri* The midpoint redox potentials were obtained from voltammograms at a scan rate of 10 mV/s Except for the temperature all other conditions were as described in Fig 2

of −0 39 mV/°C (Fig 3) Hereabove a linear decrease of −1 22 mV/°C was observed This effect is probably the result of a change in protein conformation at 18°C which influences the immediate vicinity of the iron-sulfur clusters [16] Ferredoxin showed a midpoint potential of −340 mV versus NHE at the physiological growth temperature of 37°C [20] The E_m was pH independent between pH 6–10 which indicates that the electron transfer mechanism does not involve proton exchange Grahame [21] who isolated a ferredoxin from acetate-grown *M barkeri* measured a significantly lower E_m of −420 mV at pH 8 0 This ferredoxin may be different from the one discussed here that was obtained from methanol grown cells

In conclusion we have demonstrated that the ferredoxin from methanol-grown *M barkeri* strain MS contains 2[4Fe-4S] clusters per monomer which accords the eight cysteine spacing in the amino acid sequence [1 7] The previously reported presence of a 3Fe cluster [3] could be an isolation artifact Degradation of [4Fe-4S] clusters was also found for the ferredoxin of *M thermophila* [8,9]

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The electrochemistry of 5-hydroxybenzimidazolylcobamide

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The Electrochemistry of 5-Hydroxybenzimidazolylcobamide

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Methanogenic archaea typically contain 5-hydroxybenzimidazolylcobamide (cba-HBI) as the prosthetic group of a number of methyltransferases involved in their central metabolic pathways. In this paper the (acidic) dissociation constants and standard oxidation–reduction potentials of the Co^{3+}/Co^{2+} and Co^{2+}/Co^{1+} couples of isolated aquo-cba-HBI were measured. Comparison of the data to those established for 5,6-dimethylbenzimidazolylcobamide (cobalamin) showed that the 5-hydroxybenzimidazolyl (HBI) nucleotidic base hardly affected the redox potentials. HBI, however, proved to be the weaker ligand, thus favoring "base-off" formation. The implications for the functioning of cba-HBI in biochemical methyl group transfer reactions are discussed. © 1995 Academic Press, Inc.

Key Words: **5-hydroxybenzimidazolylcobamide; cobalamin; corrinoid; cyclic voltammetry; redox potential; ligand interaction.**

Corrinoids, cobalt-containing macrocycles (Fig. 1), act as catalysts in distinct types of biochemical reactions like C-C rearrangements (1) and methyl group transfers (2, 3). The reactions take advantage of the particular chemical properties of the corrinoids that are directly related with the redox state of the central cobalt atom (2–4). The cobalt atom may occur in three oxidation states, Co(III), Co(II), and Co(I). In cob(III)amide the cobalt is six-coordinated by four equatorial nitrogens derived from the tetrapyrrole (corrin) macrocycle An axially bound nucleotide serves as the lower (α-)ligand and the upper (β-)position may be occupied by ligands like water, cyanide, a methyl, or a 5′-deoxyadenosyl group. Pentacoordinated cob(II)amide is an excellent radical trap and in the Co^{2+} redox state the corrinoid is involved in C-C rearrangements (5). The square-planar, four-coordinate cob(I)amide, one of nature's most powerful nucleophiles, acts as a catalyst in methyl group transfers (2–4)

The *N*-heterocyclic base of the nucleotide moiety is usually 5,6-dimethylbenzimidazole (DMBI)[2] (5, 6). Corrinoids present in methanogenic archaea typically contain 5-hydroxybenzimidazole (HBI) as the nitrogenous base (7). In these organisms 5-hydroxybenzimidazolylcobamide (cba-HBI; "Factor III") is the prosthetic group of a number of methyltransferases involved in the central methanogenic pathways from hydrogen and CO_2 (8, 9), methanol (10), or acetate (11, 12). Substitution of DMBI for HBI will likely affect the electrochemical properties of the corrinoid and as the consequence its catalytic action. These properties are well documented for 5,6-dimethylbenzimidazolylcobamide (cobalamin, cba-DMBI) (13–17). Since no such information was available for cba-HBI, we studied its electrochemistry The results are discussed in view of its role in biochemical methyl group transfer.

EXPERIMENTAL PROCEDURES

Materials Cyclohexylaminoethanesulfonic acid, 2-[tris(hydroxymethyl)-methylamino]ethanesulfonic acid, 3-(cyclohexylamino)-1-propanesulfonic acid, and aquo cobalamin were purchased from Sigma Chemical Co (St Louis, MO) DEAE–Sepharose-Cl-6B was from Pharmacia LKB Biotechnology A B (Uppsala, Sweden) Octadecyl C_{18} was purchased from J T Baker (Phillipsburg, NJ) and Fractogel EMD SO_3-650 (M) was obtained from Merck–Schuchardt A G (Darmstadt, Germany) Sep Pak C_{18} cartridges were from Waters Associates (Milford, MA) Tetrabutylammonium *p*-toluenesulfonate was prepared by neutralization of a 1 2 M aqueous solution of tetrabutylammonium hydroxide (Fluka Chemie AG, Buchs, Switzerland) and by *p*-toluenesulfonic acid (BDH Chemicals Ltd , Poole, England) Gasses were supplied by Hoek–Loos (Schiedam, The Netherlands) Buffers (100 mM concentrations) were prepared by adjusting solutions of the following compounds with NaOH to the indicated pH ranges phosphoric acid (pH 2–2 5 and pH 11 5–12 5), citric acid (pH 3–6 5), 2-[tris(hydroxymethyl)-methylamino]ethanesulfonic acid (pH 7–7 5), Tris (pH 8–9), cyclohexylaminoethanesulfonic acid

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[2] Abbreviations used DMBI, 5,6 dimethylbenzimidazole, HBI, 5-hydroxybenzimidazole, cba-DMBI, cobalamin, cba-HBI, 5-hydroxybenzimidazolylcobamide, SHE, standard hydrogen electrode

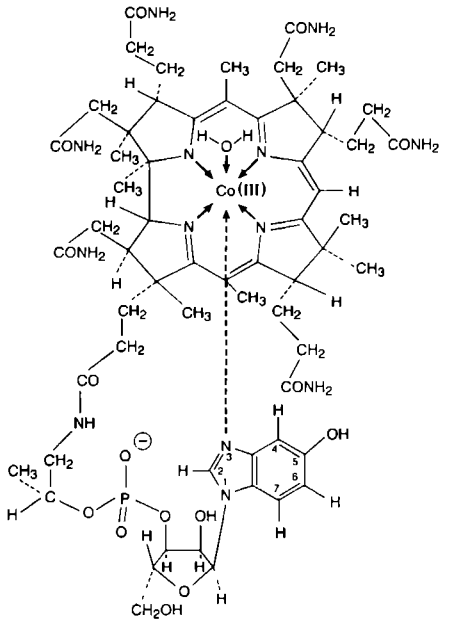


FIG. 1. Structure of Coα-[α-(5-hydroxybenzimidazolyl)]-Coβ-aquocobamide (cba-HBI)

(pH 9.5), and 3 (cyclohexylamino)-1-propanesulfonic acid (pH 10–11). A mixture of HCl and KCl was used as a buffer at pH 1–1.5. Solutions of perchloric acid, acetic acid, and sulfuric acid were used to acquire pH 0.5 and below.

Corrinoid isolation. Cba-HBI was purified from cell-free extract of *Methanosarcina barkeri* strain MS (DSM 800) as the cyano form. Organisms were grown and extracts prepared as described before (18). Extraction in hot methanol containing 0.01% KCN and subsequent purification employing DEAE–Sepharose Cl-6B, octadecyl C_{18}, and Fractogel EMD SO_3-650 (M) column chromatography proceeded essentially according to Pol *et al.* (19). After each ion chromatography step CN-cba-HBI-containing fractions were combined, concentrated, and desalted with Sep-Pak C_{18} cartridges. For the preparation of the cob(II)amide and of aquo-cob(III)amide, pure CN-cba-HBI was reduced by addition of 25 μl of hydrogenase from *M. barkeri* (0.12 μmol benzylviologen reduced min^{-1} ml^{-1}) (18) followed by incubation under a hydrogen atmosphere for 48 h at 37°C. Thereafter the cob(II)amide was applied under strict anaerobic conditions to a Sep-Pak C_{18} cartridge. Following thorough washing with distilled water, the corrinoid was eluted from the cartridge with 80% methanol. Aquo-cba-HBI was obtained in 92% yield by exposure of cob(II)amide to air and subsequent purification on a Fractogel EMD SO_3-650 (M) column. The nucleoside of cba-HBI, 5-hydroxy-1-α-ribofuranosylbenzimidazole, was obtained by hydrolysis of CN-cba-HBI with cerous hydroxide and purified as described by Pol (20).

Analytical procedures. Ultraviolet–visible absorption spectra in the above-mentioned buffers were recorded at 22°C using an Hitachi U-3200 spectrophotometer. Direct electrochemistry (cyclic voltammetry) of 20 μl amounts of corrinoid solution (0.1 or 0.3 mM) took place at 22°C under an argon atmosphere using a nitric acid treated glassy carbon, a platinum, and a saturated calomel three-electrode system. Methodology and microapparatus were as described by Hagen (21). Redox potentials as a function of the pH were determined by diluting (1:1) the corrinoid stock solution with buffer of the desired pH. Reactant adsorption was prevented by the presence of 50 mM tetrabutylammonium *p*-toluenesulfonate (15).

RESULTS

Dissociation Constants of 5-Hydroxybenzimidazolylcobamide in the Different Oxidation States

The pK_a values were derived from the pH-dependent changes in the uv–visible light spectra of aquo-cob(III)amide-HBI and of the cob(II)amide (Table I). The former compound showed three pK_a values. The pK_a = 7.8 ± 0.2 and pK_a = 9.7 ± 0.2 closely agreed the values reported by Pol *et al.* (22) and are assigned to the aquo- and hydroxo-cba-HBI equilibrium and to the deprotonation of the 5-OH group in HBI, respectively. At pH < −1 an additional pK_a = −1.3 ± 0.2 was found. This pK_a value reflects the association/dissociation of the nucleoside with the central cobalt, the so-called "base-on"/"base-off" equilibrium. The pH-dependent uv–visible light spectra of cob(II)amide are shown in Fig. 2. Here we measured two pK_a values (Table I). As above, the high pK_a = 9.8 ± 0.4 was attributed to the ionization of the HBI phenolic group. The lower pK_a = 3.6 ± 0.1 was assigned to the base-on/base-off equilibrium. The spectrophotometric determination of the ionization constants of Co(I)-cba-HBI was hampered by its extreme oxygen sensitivity. Since in this oxidation state the corrinoid is exclusively present in the uncoordinated, base-off state (23), the pK_a values of isolated 5-hydroxy-1-α-ribofuranosylbenzimidazole were considered a good approximation of the pK_a values of the base bound to the cob(I)amide. Two pH-dependent shifts in the uv–visible spectrum of the isolated nucleoside were found showing a pK_a = 10.1 ± 0.1 and a pK_a = 5.25 ± 0.1 which were assigned to the (de)protonation of the 5-OH group and of N(3) of HBI, respectively (6, 20).

Reduction of the Cob(III)amide to Cob(II)amide

Cyclic voltammetry was used for determination of the standard potential of reduction for the Co(III)/Co(II) redox couples of cba-HBI as a function of pH. Reduction of the Co(III) corrinoid was a slow reaction but at a scan rate of 4 mV/s the n = 1 electron transfer was fully reversible, as judged from the reproducible cathodic-to-anodic peak potential separation of 64 ± 3 mV (Fig. 3A). A minor distortion around 375 mV is observed in the voltammogram presented in the figure, but this was also found in the absence of corrinoid and therefore ignored. The response was optimal in the presence of 50 mM tetrabutylammonium *p*-toluenesulfonate. The redox potentials were measured over a pH range varying between 2 and 12. Between pH 3.5 and

TABLE I

Ultraviolet–Visible Light Spectral Data of 5-Hydroxybenzimidazolyl-cob(III)amide and -cob(II)amide

Compound	pH	Extinction maxima and coefficients nm ($\epsilon \times$ mM^{-1} cm^{-1})	pK_a values
Cob(III)amide			
H_2O-cba-HBI	−1 6	540(12 0), 512(sh)[a](10 6), 416(5 0), 358(26 0) 276(sh)(24 3)	−1 3 ± 0 2
	6 0	522(8 9), 497(8 4), 410(3 7), 352(27 0), 290(sh)(16 8), 274(18 5), 258(sh)(19 3)	7 8 ± 0 2
HO-cba-HBI	9 0	532(9 7), 510(sh)(9 1), 418(4 2), 357(21 4), 300(sh)(14 8), 280(16 4)	9 7 ± 0 2
	12 0[b]	535(9 9), 510(sh)(9 2), 418(4 4), 356(22 0), 325(18 3), 280(sh)(12 9)	
Cob(II)amide			
Cba-HBI	2 0	470(9 6), 411(sh)(6 6), 316(22 8), 290(21 2), 267(23 1)	3 6 ± 0.1
	7 0	474(9 4), 407(7 9), 312(27 7)	9 8 ± 0 4
	11 5	587(sh)(2 5), 526(sh)(5 2), 476(8 5), 405(6 9) 362(sh)(10 8) 313(25 7)	

[a] sh, shoulder

[b] These values were taken from Ref 19 and used to calculate the other ϵ values

7 a constant $E^0 = 226 \pm 9$ mV vs the standard hydrogen electrode (SHE) was observed. Below pH 3 and between pH 8 and 9 E^0 varied as a function of pH, whereas above pH 10 the redox potential remained constant at $E^0 = 130 \pm 11$ mV vs SHE (Fig. 4).

Reduction of the Cob(II)amide to Cob(I)amide

Measurements of the Co^{2+}/Co^{1+} redox couple used the aquo-cob(III)amide as the starting material. Prior to the voltammetric run, the compound was quantitatively reduced to the Co(II) level at the initial potential of the scan (−150 mV vs SHE) (15). At a scan rate of 50 mV/s the one-electron transfer reaction was fully reversible over the pH range tested, as judged from the cathodic/anodic peak potential separation of 59 ± 2 mV (Fig 3B). This fast scan rate allowed the determination of the redox potential of the base-on cob(II)amide/cob(I)amide couple without interference of the base-on/base-off reaction, which can precede the actual reduction of Co^{2+} at low scan rates (13, 14) The electrode response was independent of 50 mM tetrabutylammonium *p*-toluenesulfonate. The experiments were conducted at pH values ranging between 2 and 12. The E^0 values thus determined are shown in the lower part of Fig 4 E^0-stable regions occurred around pH 2–3 ($E^0 = -500 \pm 3$ mV) and between pH 5 5 and 10 ($E^0 = -592 \pm 2$ mV). Above pH 10 a third region may be present with a slightly, but not significantly, lower $E^0 = -598 \pm 11$ mV vs SHE. Below pH 2.5 the voltammogram showed a catalytic character and simultaneously gas bubbles appeared in the sample. This was due to the formation of the cobalt hydride by protonation of cob(I)amide-HBI and its decomposition into the cob(II)amide and molecular hydrogen (13, 24). Because this reaction interfered in the determination of the redox potentials of the Co^{2+}/Co^{1+} couple, no data could be collected at pH values below 2 5.

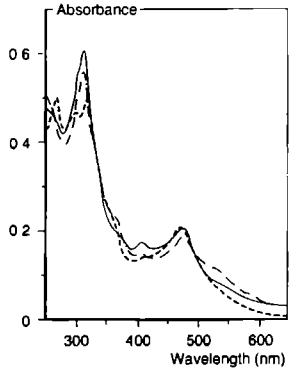


FIG. 2. Ultraviolet–visible light spectra of 0 022 mM 5-hydroxybenzimidazolylcob(II)amide recorded at pH 2 0 (---), pH 7 0 (——), and pH 11.5 (– · –)

DISCUSSION

In this study we measured the acidic dissociation constants of cba-HBI as well as the standard redox potentials as a function of pH of the Co^{3+}/Co^{2+} and the Co^{2+}/Co^{1+} couples. The results are summarized in the E^0–pH diagram shown in Fig. 4. In the figure the voltammetrically determined redox potentials could be fitted into pH-invariant and pH-dependent sections bounded by the spectrophotometrically established pK_a values (13, 14). The diagram provides the information for thermodynamic calculations of all possible reactions. It allows an easy comparison of the electrochemical properties of cba-HBI and those of the ubiquitously occurring cobalamin, or between free and enzyme-bound cba-HBI.

The presence of HBI affects the electrochemistry of cba-HBI in relation to cobalamin in a number of aspects (Table II). The 5-OH group introduces an additional

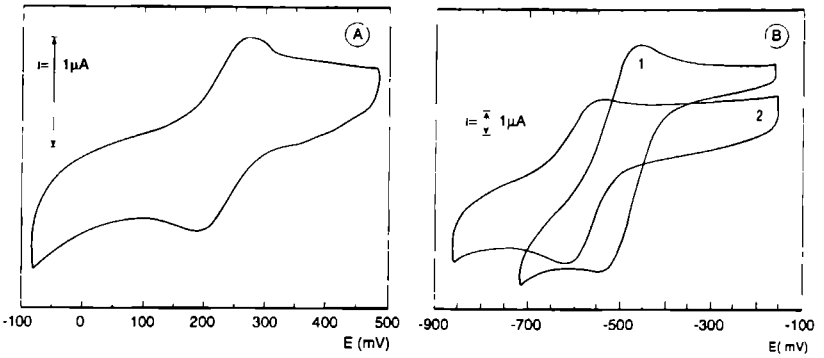


FIG. 3. Cyclic voltammograms of (A) the Co^{1} /Co^{2+} and (B) the Co^{2+} /Co^{3+} couples of cba-HBI Experimental conditions (A) H_2O–cba-HBI (20 μl, 0 1 mM) in 50 mM sodium citrate buffer (pH 6 0) containing 50 mM tetrabutylammonium *p*-toluenesulfonate, potential scan rate, 4 mV/s, temperature, 22°C (B) Aquo-cba-HBI (20 μl 0 3 mM) in 50 mM Tris/Cl buffer, pH 8 0 (1), or 50 mM sodium citrate buffer, pH 3 0 (2), potential scan rate, 50 mV/s, temperature, 22°C In Fig 3B voltammograms 1 and 2 show the response of the "base-on" Co^{2+} /Co^{1} and "base-off" Co^{2} /Co^{2} couple, respectively The potential axis is defined versus the standard hydrogen electrode

ionizable function The pK_a(5-OH) was about constant among the different redox states of the central cobalt atom, indicating that coordination to the corrin nucleus only slightly influences this ionization However, since the 5-hydroxy function in HBI is a better electron donor than the 5-methyl group in DMBI the protonation constant of the N(3) of the base, as well as the base-on/base-off equilibrium constants, was significantly higher (Table II). Remarkably, the HBI base had only little effect on the redox potentials of the cob(III)amide/cob(II)amide and cob(II)amide/cob(I)amide couples Reduction of the base-on cba-HBI species took place at values 10–12 mV higher than those of the respective cobalamins HBI, being the weaker base, thus, somewhat facilitates the reduction of the cobalt ion. As expected, reduction of the base-off species occurred at nearly identical E^0 values, since cba-HBI and cobalamin contain structurally the same corrin nucleus

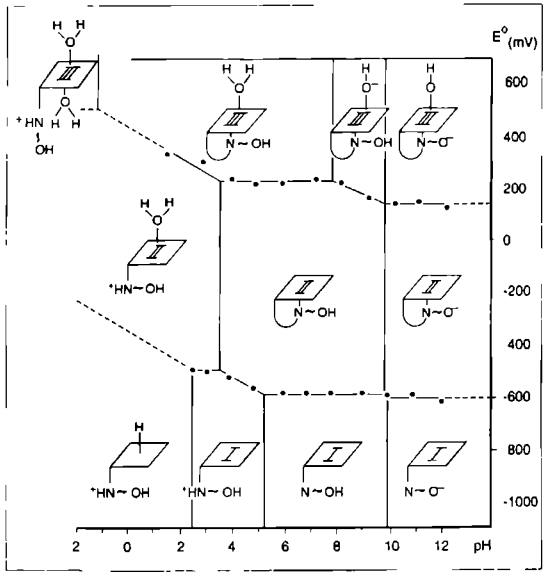


FIG. 4. Standard redox potential–pH diagram of the Co^{1} /Co^{2} and Co^{2} /Co^{1} couples of cba-HBI at 22°C The corrin moiety of cba-HBI is schematically represented by the square plane with the formal redox state of the cobalt indicated by roman numbers, the N-(3) and 5-hydroxy group of the HBI-base are illustrated by N~OH Dashed lines represent areas of theoretical redox potentials assuming a $\Delta E^{0}/\Delta pH = -59$ mV/pH

In view of the relatively subtle differences in the chemical properties with respect to cobalamin, one now might ask which advantage methanogenic bacteria could derive from the specific presence of cba-HBI in their metabolism. Indeed, when growth medium of *Methanobacterium thermoautotrophicum* was supplemented with DMBI, the compound was incorporated as cobalamin, without a notable effect on the growth rates and methanogenic capacities (26). Methanogenic bacteria, thus, could simply lack the possibilities of DMBI biosynthesis From a chemical point of view, a more positive answer cannot be ruled out. In methanogens and other anaerobic microorganisms, cba-HBI is the prosthetic group of a number of methyltransferases involved in the central metabolism (8–12, 27). Superreduced cob(I)amide is the catalytically active species and methyl-cob(III)amide is an intermediate in the subsequent methylation and demethylation steps (2–4, 10, 18, 28) In the chemical and biochemical transmethyla-

TABLE II

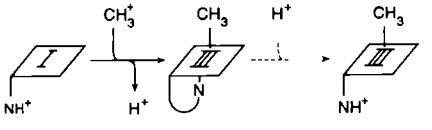
Comparison of the Electrochemical Properties between 5-Hydroxybenzimidazolylcobamides and Cobalamins

Compound	pK_a values		E^0 values (mV)			Refs
	pK_a(N-3)/ "Base-on/off"	pK_a(5-OH)	"Base-off"	"Base-on"	Hydroxo	
Co(III) (→ Co(II))						
Cba-HBI	−1 3 ± 0 2	9 7 ± 0 2	516[a]	226 ± 9	130 ± 11	This paper
Cobalamin	−2 4		515	208,	208 -	11, 13, 14
				216 ± 8[b]	59/pH	16, 25
Co(II) (→ Co(I))						
Cba-HBI	3 6 ± 0 1	9 8 + 0 4	−500 + 3	−592 + 2	600 + 4	This paper
Cobalamin	2 7		−494	−604	-604	13, 14
Co(I)[c]						
Cba-HBI	5 25 ± 0 1	10 1 + 0 1				This paper
Cobalamin	4 7					11

[a] The value was calculated by extrapolation of the data shown in Fig 4
[b] Measured by cyclic voltammetry (P Daas, unpublished results)
[c] The pK_a values are those measured for the isolated nucleosides

tions, base-on/base-off interconversions are of key importance (Scheme 1) Compared to cobalamin, cba-HBI apparently favors base dissociation, *without* necessarily negatively effecting base coordination Methylation of (base-off) cob(I)amide yielding (base-on) methyl-cob(III)amide takes advantage of nucleotide coordination, which stabilizes the Co–C bond formed (4, 29). This so-called thermodynamic *trans*-effect is determined by the difference in pK_a values between the N(3) of the uncoordinated base and the base-on/base-off equilibrium constant of the methylated cobamide. Despite the individual differences in the constants (22, 30), nearly identical ΔpK_a values are found for cba-HBI and cobalamin methylation (Scheme 1). Conversely, the *trans*-effect will stabilize the Co–C bond against heterolytic cleavage during the methyl group displacement, and the preceding formation of the base-off methyl derivative will facilitate Co–C bond cleavage (4, 29). Due to the higher pK_a, base dissociation in methyl-cba-HBI is clearly favored with respect to methylcobalamin

From the above discussion it could be concluded that cba-HBI seems to be better adapted to a specific role in transmethylation reactions However, it should be stressed that the considerations apply to aqueous solutions of the corrinoids Electrochemical and base-off/base-on properties may be altered within a protein environment. For few enzymes that contain HBI or the structurally related 5-methoxybenzimidazole as the nitrogenous base, oxidation–reduction potentials were actually measured (11, 12, 31). In the enzymes, cob(II)amide was specifically present either in the base-on ($E^0(Co^{2+}/Co^{1+}) = -630$ mV) (31) or thermodynamically unfavorable base-off state (E^0 = - 486 and −504 mV) (11, 31), thus showing standard redox potentials that were close to those measured here for the respective free cba-HBI derivatives (Table II). However, a cba-HBI-containing enzyme isolated from *M barkeri* involved in acetyl-CoA cleavage and having an as yet undefined base configuration had a significantly higher $E^0(Co^{2+}/Co^{1+}) = -426$ mV vs SHE (12) Our data pre-



	pK_a (N-3)	pK_a (on/off)	ΔpK_a	(ΔG_{stab} kJ/mol)
cba-HBI	5 25	3 3	1 95	(-11 1)
cobalamin	4 7	2 7	2 0	(-11 4)

SCHEME 1

dict that this may be achieved by keeping the corrinoid in the base-off state Substitution of water by a less nucleophilic fifth ligand or a weakening of the aquo–cobalt bond by hydrogen bridges then would contribute to a further increase of the redox potential

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Purification and properties of an enzyme involved in the ATP-dependent activation of the methanol:2-mercapto-ethanesulfonic acid methyltransferase reaction in *Methanosarcina barkeri*

Piet J. H. Daas, Pierre Willemsen, René J. Theunissen, Jan T. Keltjens, Chris van der Drift, and Godfried D. Vogels

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Abbreviations used: CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate; CH_3-S-CoM, methyl-coenzyme M, 2-(methylthio)ethanesulfonic acid; HS-CoM, coenzyme M, 2-mercapto-ethanesulfonic acid; IEF, isoelectric focusing; MAP, methyltransferase activation protein; MT_1, methanol:5-hydroxybenzimidazolylcobamide methyltransferase; MT_2, Co-methyl-5-hydroxybenzimidazolylcobamide: HS-CoM methyltransferase; PAGE, polyacrylamide gel electrophoresis; TES, *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid; TLC, thin-layer chromatography.

Purification and properties of an enzyme involved in the ATP-dependent activation of the methanol:2-mercaptoethanesulfonic acid methyltransferase reaction in *Methanosarcina barkeri*

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SUMMARY

In *Methanosarcina barkeri* transfer of the methyl group of methanol to 2-mercaptoethanesulfonic acid is catalyzed by the concerted action of two methyltransferases. The first is the corrinoid-containing methanol: 5-hydroxybenzimidazolylcobamide methyltransferase (MT_1), which binds the methyl group of methanol to its corrinoid prosthetic group. MT_1 is only catalytically active when the cobalt atom of the corrinoid is present in the highly reduced Co(I) state. In the course of its purification and even during catalysis MT_1 becomes oxidatively inactivated. The enzyme, however, may be reductively reactivated by a suitable reducing system (hydrogen and hydrogenase), ATP and an enzyme called methyltransferase activation protein (MAP). To elucidate the role of ATP in this reactivation process, MAP was purified to apparent homogeneity. The protein, M_r = 60,000, was shown to interact with ATP during the activation of MT_1. MAP was readily autophosphorylated by [γ-^{32}P]ATP. From this it was concluded that phosphorylated MAP is the actual effector in the activation of MT_1. This paper is the first to report on the presence, isolation, and function of a phosphorylated protein in a methanogenic archaeon.

Corrinoid (B_{12})-containing enzymes catalyze two distinct classes of reactions, notably C-C rearrangements and methyl group transfers. The typical example of the latter enzymes is methyltetrahydrofolate:homocysteine methyltransferase (methionine synthase) (1). In recent years evidence has been accumulating that corrinoid proteins play a central role in a variety of anaerobic conversion reactions like acetate biosynthesis and degradation, biodegradation of methoxylated aromatic compounds, methylated amines and sulfur compounds, and methylated

heavy metals (2,3,4). The conversions are brought about by diverse groups of obligate anaerobic micro-organisms, including sulfate reducers, homo-acetogens and obligate proton reducers, and methanogenic archaea (3,4). Methylotrophic methanogens specialized in methane formation from methylated one-carbon compounds are particularly rich in corrinoid proteins (2).

Being nature's most powerful nucleophile the corrinoid prosthetic group is elegantly designed to act in methyl group displacement (4). The coenzyme, however, suffers from the intrinsic disadvantage that this function is only displayed, when the central cobalt is present in the superreduced Co(I) reduction state and even the slightest of oxidative *in vivo* and *in vitro* conditions cause an oxidation of the cobalt atom and the concomitant enzyme inactivation. For this reason, organisms that employ corrinoid proteins in their metabolism contain certain reactivation systems in which ATP is somehow used to push the unfavorable Co(II) to Co(I) reduction across a thermodynamic barrier (3,5). In the methionine synthase reaction ATP functions as the substrate in the synthesis of a potent methylated trapping agent, S-adenosyl-methionine (5). The action mechanism of ATP in the reductive activation of other methyltransferases present in the obligately anaerobic micro-organisms mentioned above is, however, unknown. To clarify the role of ATP we now investigated the methanol:2-mercaptoethanesulfonic acid (coenzyme M, HS-CoM) methyltransferase system from the methylotrophic methanogen *Methanosarcina barkeri.*

Methanol:HS-CoM methyltransferase catalyzes the synthesis of methyl-coenzyme M (CH_3-S-CoM), which is the substrate of the methane-forming reaction (2). The methyltransferase is a multicomponent system composed of two methyltransferases, methanol: 5-hydroxybenzimidazolylcobamide methyltransferase (MT_1) and Co-methyl-5-hydroxybenzimidazolylcobamide:HS-CoM methyltransferase (MT_2). MT_1 accepts the methyl group of methanol and binds it to the oxidation-sensitive 5-hydroxybenzimidazolylcob(I)amide prosthetic group. MT_2 subsequently transfers the methyl group to HS-CoM (6,7). Since MT_1 can only be isolated in an oxidized state (7), the presence of a suitable reducing system (hydrogen and hydrogenase) in the assays is a prerequisite for the recovery of MT_1 activity (8,9). The electron carrier ferredoxin enhances the activation rate, but the compound is not obligatorily required (10). Activation is strictly dependent on the presence of catalytic amounts of ATP and of an enzymic component(s) present in crude cell extracts termed Methyltransferase Activation Protein (MAP) (10). In this paper we describe the first-time purification of MAP and show it to be the primary site of action of ATP. Evidence

will be presented that MAP is autophosphorylated by ATP suggesting that phosphorylated MAP is the effector in MT_1 activation.

MATERIALS AND METHODS

Cell material. Mass culture of cells of *M. barkeri* strain MS (DSM 800) and the anaerobic preparation of cell extract proceeded as described before (10,11).

Enzyme Assays. Unless stated otherwise, incubation mixtures were prepared in an anaerobic glove box. Reactions were performed in 10-ml serum vials closed with butyl rubber stoppers and aluminum crimp seals.

MAP activity was determined by the ability to restore the methanol-dependent HS-CoM conversion to CH_3-S-CoM when added to a reaction mixture containing MT_1, MT_2/hydrogenase, and ferredoxin. The enzymes were obtained by DEAE-Sepharose-Cl-6B chromatography of cell extract of *M. barkeri* (10). A typical reaction mixture (final volume, 100 µl) contained 50 mM *N*-tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES/K^+) buffer (pH 7.0), 24 mM $MgCl_2$, 10 mM methanol, 10 mM HS-CoM, 2 mM ATP, 1 mM 2-bromoethanesulfonic acid (to inhibit enzymic reduction of CH_3-S-CoM in crude systems), 20 µl MT_2/hydrogenase fraction (2.47 mg protein ml^{-1}; 1.42 µmol HS-CoM converted min^{-1} mg $protein^{-1}$; 0.10 µmol benzylviologen reduced min^{-1} mg $protein^{-1}$; 0.02 µmol coenzyme F_{420} reduced min^{-1} mg $protein^{-1}$), 12.5 µl MT_1 fraction (3.43 mg protein ml^{-1}; 0.88 µmol HS-CoM converted min^{-1} mg $protein^{-1}$), 5 µl ferredoxin fraction (0.36 mg protein ml^{-1}; 0.34 µmol dithiodiethanesulfonic acid reduced min^{-1} mg $protein^{-1}$), and 40 µl column fraction as a source of MAP. After gassing with 50% H_2/50% N_2 (100 kPa) the vials were kept on ice. Reactions were started by placing the vials at 37°C. After appropriate incubation periods, usually 0, 15, 30, 45, and 60 min, reactions were stopped by placing the vials on ice. Hereafter, the amount of HS-CoM present in the incubation mixtures was measured with Ellman reagent (see below) and activity of methyl group transfer from methanol to HS-CoM was calculated from the rate of HS-CoM conversion (10).

Since MAP only affects the lagging of the methyltransferase reaction, rather than the rate of HS-CoM conversion, a nonlinear relation exists between the amount of MAP present in the assays and the amount of HS-CoM converted at a certain point of time (10). To quantify MAP activity an arbitrary unit was defined to enable a purification scheme to be drawn up. One arbitrary unit (AU) was defined as the amount of MAP present in 5 µl of cell extract (0.13 mg protein). The latter value was chosen such that HS-CoM conversion by the extract alone was negligible, while when added to the standard assay the reaction took off without appreciable lagging; under these conditions 0.38 µmol HS-CoM was converted in 45 min. To determine the MAP activity of a purified fraction, a series of standard reaction mixtures was prepared with decreasing amounts of the fraction. Following an incubation of 45 min, the particular test mixture which just showed a HS-CoM conversion equal to 5 µl cell extract, by definition contained 1 AU of MAP activity.

The enzymatic activities of MT_1 and MT_2 were determined by measuring the rate of HS-CoM decrease in the presence of a saturating amount of MAP (routinely 25 µl MAP fraction obtained after DEAE-Sepharose chromatography of cell extract

(10)). The methyltransferase activities obtained were linearly dependent on the amount of MT_1 or MT_2/hydrogenase fraction added. Hydrogenase and ferredoxin activity were determined as described before (8,10).

MAP-dependent ATP conversion was followed in reaction mixtures that were essentially the same as described for the MAP activity assay, except that 0 125 μM ATP was present; protein was added as specified in the text. After incubation periods of 0, 30, and 60 min reactions were stopped by placing the vials on ice and samples were drawn for analysis. Hydrolysis of [8-^{14}C]ATP was examined in reaction mixtures (final volume, 100 μl) containing 50 mM TES/K^+ buffer (pH 7.0), 2 μM $MgCl_2$, 2 μM [8-^{14}C]ATP (1.89 TBq mol^{-1}), and 40 μl of protein fraction. Incubation took place for 0, 15, 60, and 120 min at 37°C under 50% H_2/50% N_2 (100 kPa). By the same procedure the conversion of [γ-^{32}P]ATP was followed except that 2 μM [γ-^{32}P]ATP (8350 TBq mol^{-1}) was used.

Purification of MAP. Because MAP is an oxygen-labile protein (10), all handlings were performed at room temperature in an anaerobic glove box (97 5% N_2/ 2.5% H_2). The purification procedure started by applying 10 ml of cell extract to a DEAE-Sepharose Cl-6B column and separating MAP from the other proteins involved in the methanol HS-CoM methyltransferase reaction as described before (10) Fractions containing MAP activity eluting between 0 25 and 0 34 M NH_4Cl were combined and concentrated to 6 ml by Amicon YM-10 ultrafiltration. To this fraction 6 ml of 50 mM TES/K^+ buffer (pH 7.0) containing 1 mM dithiothreitol and 4 M NH_4Cl was added The sample was placed on a column packed with Phenyl-Sepharose CL-4B (6.5 by 1 5 cm) and equilibrated in 50 mM TES/K^+ buffer (pH 7 0) containing 2 M NH_4Cl, 15 mM $MgCl_2$, and 1 mM dithiothreitol After application of the sample, the column was eluted with 70 ml of equilibration buffer followed by single step elution of bound protein with 50 ml of 50 mM TES/K^+ buffer (pH 7 0) containing 15 mM $MgCl_2$, 1 mM dithiothreitol, and 10% ethylene glycol The eluate was monitored at 280 nm and 2.5-ml fractions were collected at a flow rate of 3 ml min^{-1}. MAP activity was eluted at the 10% ethylene glycol step Active fractions were combined and concentrated by Amicon YM-10 ultrafiltration to a final volume of 1 ml MAP was further purified using a Perkin-Elmer fast protein liquid chromatography system placed inside the glovebox and equipped with a TSK DEAE-5-PW column (7 5 cm by 0.75 cm) The column was equilibrated in 50 mM TES/K^+ buffer (pH 7 0) containing 15 mM $MgCl_2$, 1 mM dithiothreitol, 10% ethylene glycol, and 0 5 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) After application of the sample, separation took place by a 7-ml wash with the equilibration buffer followed by a 100-ml linear gradient of 0 to 0 5 M NH_4Cl in the buffer Fractions of 0 9 ml were collected at a flow rate of 0 45 ml min^{-1} MAP activity was recovered in a peak that was eluted at 0.16 M NH_4Cl. Fractions containing MAP were combined and concentrated by Amicon YM-10 ultrafiltration to a volume of 1 ml Hereafter, MAP was brought to homogeneity on a TSK-Gel HA-1000 column (7 5 by 0 75 cm) equilibrated in 50 mM TES/K^+ buffer (pH 7 0) containing 1 mM dithiothreitol, 0 5 mM CHAPS, and 10% ethylene glycol The sample was applied to the hydroxylapatite column and the column was washed with 15 ml of the equilibration buffer Bound protein was eluted by a 50-ml simultaneous linear gradient of 50 to 0 mM TES/K^+ buffer (pH 7 0) and 0 to 300 mM ammonium phosphate buffer (pH 7 0), both buffers contained 1 mM dithiothreitol, 0 5 mM CHAPS, and 10% ethylene glycol Fractions of

0.9 ml were collected at a flow rate of 0.45 ml min^{-1}. MAP was present in a small symmetrical peak that was eluted at 145 mM ammonium phosphate and 26 mM TES/K^+. The enzyme pool was concentrated and desalted on an Ultrafree-MC filter unit (nominal molecular weight cutoff of 10,000) by washing with 50 mM TES/K^+ buffer (pH 7.0) containing 1 mM dithiothreitol and 10% ethylene glycol.

Photoaffinity labeling. Reaction mixtures containing the protein fraction (560 µl) and 2 mM 8-azido-ATP were prepared inside the anaerobic glove box in 1-ml quartz cuvettes. The cuvettes were closed with rubber stoppers, taken out of the box, and gassed with nitrogen (30 kPa). The reaction mixtures were illuminated for 1 hour at 4°C by a 6 Watt 5C UV-light (Philips, Eindhoven, the Netherlands) positioned at a distance of 6 cm. Hereafter, the mixtures were thoroughly washed with 50 mM TES/K^+ buffer (pH 7.0) containing 1 mM dithiothreitol and 10% ethylene glycol inside the anaerobic glove box on Ultrafree-MC filter units to remove noncovalently bound nucleotides. Activity of the proteins was measured as described above. The cuvettes and vials of the controls containing the unexposed protein fractions were subject to the same procedure except that they were wrapped in aluminum foil.

Analytical procedures. Native polyacrylamide gel electrophoresis (PAGE), denaturing SDS-PAGE, and isoelectric focusing (IEF) were performed with prefabricated minigels using the Pharmacia PhastSystem equipment (Uppsala, Sweden). The gels were stained with Coomassie Brilliant Blue R-250. The molecular mass of denaturated protein was determined by electrophoresis on a 10-15% gradient minigel with SDS-buffer strips. Prior to application the protein was stored at room temperature (22°C) for 1 hour in 5 mM Tris/Cl^- (pH 8.8) that contained 1% SDS, 4% β-mercaptoethanol, and 10% glycerol. Protein markers (Biorad Laboratories, Richmond, CA, USA) were the following (Da): α-lactalbumin (14,400), soybean trypsin inhibitor (21,500), bovine carbonic anhydrase (31,000), hen egg white lysozyme (45,000), bovine serum albumin (66,200), and rabbit muscle phosphorylase *b* (97,400). Native PAGE was performed on a 8-25% gradient minigel using the following markers (Sigma, St. Louis, MO, USA): α-lactalbumin (14,200), chicken egg albumin (45,000), the monomer (66,000) and dimer (132,000) of bovine serum albumin, and the trimer (272,000) and hexamer (545,000) of jack bean urease. Isoelectric focusing was performed with a pH 3-9 IEF-gel using the pI 4.6-9.6 IEF standard proteins from Biorad (Biorad Laboratories, Richmond, CA, USA). The molecular mass of the native protein was also estimated by gel filtration on Superose-6 HR (30 by 1 cm) calibrated with cyanocobalamin (1,355) and the following high molecular mass markers (Da) obtained from Sigma (St. Louis, MO, USA): carbonic anhydrase (29,000), bovine serum albumin (66,000), apoferritin (443,000); Blue dextran was added for determination of the void volume. The column was equilibrated and eluted with 50 mM TES/K^+ buffer (pH 7.0) containing 15 mM $MgCl_2$, 1 mM dithiothreitol, 10% ethylene glycol, 150 mM NH_4Cl, and 0.5 mM CHAPS. Fractions of 0.36 ml were collected at a flow rate of 0.18 ml min^{-1}.

ATP concentrations were measured on a LKB-Wallac 1250 luminometer using the ATP bioluminescence CLS reagent from Boehringer (Mannheim, FRG). A 25 µl-sample of the reaction mixture was mixed with 25 µl ATP bioluminescence reagent and 450 µl 40 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-(2-ethanosulfonic acid) buffer (pH 7.75) containing 4 mM EDTA, and immediately placed in the luminometer. Determination of [8-^{14}C]ATP conversion started by adding 400 µl of 96% ethanol to

the reaction mixture at 4°C Protein and part of the [8-^{14}C]ATP was subsequently removed by centrifugation for 15 min at 16,000 x *g* (Eppendorf) 40 µl-Aliquots of the supernatant were spotted on polyethyleneimine-cellulose F thin-layer chromatography (TLC) plates (0 1 mm, Merck AG, Darmstadt, FRG) that were developed with 2 M formic acid containing 0 5 M LiCl (12) R_f values of ATP, ADP, and AMP were 0 12, 0 68, and 0 91, respectively Radioactive spots were located by autoradiography by exposing the TLC plates to Kodak XAR-5 X-ray film for 8 days at -80°C Radioactivity was quantified by cutting the TLC plates, scratching off the silica gel, and suspending the labeled compound in 1 ml of water and 9 ml of Lumagel scintillation fluid (Lumac, Schaesberg, The Netherlands) PAGE gels that were used to determine the binding of $^{32}P_i$ with protein were dried prior to analyses Hereafter, the front was cut off to remove excess unreacted [γ-^{32}P]ATP and radioactivity was located by autoradiography for 6 hours using Kodak XAR-5 X-ray film

Protein was determined with the Biorad protein reagent (Biorad Laboratories, Richmond, CA, USA) with bovine serum albumin as a standard UV-visible light absorption spectra were recorded on a Hitachi U-3200 spectrophotometer HS-CoM was determined by the method of Ellman (13) Samples of 25 µl were mixed with 3 ml 0 48 mM 2,2'-dinitro-5,5'-dithiobenzoic acid in 150 mM Tris/Cl buffer (pH 8 0) and measured immediately at 412 nm The acid stability of the phosphate linkage of the ^{32}P-labeled protein was determined as described by Martensen (14)

Materials. All chemicals used were of analytical grade HS-CoM, 2-bromoethanesulfonic acid, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanosulfonic acid), TES, and 8-azido ATP were purchased from Sigma Chemical Co (St Louis, MO) Dithiothreitol was from Serva Feinbiochemica (Heidelberg, Germany) LiCl was obtained from Merck AG (Darmstadt, FRG) ATP and CHAPS were purchased from Boehringer (Mannheim, Germany) DEAE-Sepharose Cl-6B, Phenyl-Sepharose CL-4B, and Superose-6 HR were from Pharmacia LKB Biotechnology A B (Uppsala, Sweden) TSK DEAE-5-PW and TSK-Gel HA-1000 columns were obtained from TosoHaas (Stuttgart, Germany) Ultrafree-MC filter units were acquired from Millipore (Etten-Leur, The Netherlands) [8-^{14}C]ATP (1 89 TBq mol^{-1}) came from Amersham (Buckinghamshire, UK) [γ-^{32}P]ATP (8350 TBq mol^{-1}) was obtained from ICN Biomedicals (Zoetermeer, The Netherlands) Gasses were supplied by Hoek-Loos (Schiedam, The Netherlands) To remove traces of oxygen, H_2-containing gasses were passed over a BASF RO-20 catalyst at room temperature and nitrogen was passed over a prereduced BASF R3-11 catalyst at 150°C The catalysts were a gift of BASF Aktiengesellschaft (Ludwigshafen, Germany)

RESULTS

Purification of MAP. Methyltransferase Activation Protein was purified to homogeneity from cell extract of *M. barkeri* strain MS as summarized in Table 1 After this stage only one band could be detected after native and SDS-PAGE (Fig. 1) The purification of MAP proved to be quite difficult. The aerobically unstable enzyme (10) readily inactivated upon handling and the enzyme interacted with other

Table 1. *Purification of Methyltransferase Activation Protein from M.barkeri.* The purification procedure started from 10 ml of cell extract. Enzyme assays were performed as described under "Materials and Methods". The activity is expressed in arbitrary units (AU). One AU is defined as the amount of MAP activity present in 5 µl cell extract as described under "Materials and Methods".

Step	Total protein	Total activity	Specific activity	Factor	Recovery
	mg	*AU*	*AU/mg*	*-fold*	*%*
Cell extract	264	2000	7.6	1.0	100
DEAE-Sepharose	26	363	14	1.8	18
Phenyl-Sepharose	5.9	110	19	2.5	5.5
TSK DEAE	1.6	45	28	3.7	2.3
Hydroxylapatite	0.04	24	600	79	1.2

proteins during column chromatography which resulted in poor resolution of MAP activity. The instability is reflected in the purification procedure which was accompanied with considerable losses in activity. After the first step only 18% of MAP activity was recovered. Addition of 10% ethylene glycol to the buffers, which previously was found to protect crude enzyme preparations from inactivation (10), in the purification steps following the Phenyl-Sepharose column could not prevent that a final yield of only 1.2% was found. In the final two steps of the purification procedure 0.5 mM of the zwitterionic detergent CHAPS was added to the buffers, which resulted in a better resolution of MAP activity. During SDS sample preparation MAP exhibited an unusual temperature instability. After incubation of MAP for 5 min at 80°C in SDS sample buffer a smear was observed on the gel from M_r = 60,000 and below. A same smear occurred after pretreatment in SDS sample buffer at temperatures up to 22°C (room temperature). However, when MAP was stored for one hour at room temperature in SDS sample buffer only a single band was detected with M_r = 60,000 (Fig. 1 and 2*A*). From nondenaturing 8-25% PAGE a molecular mass of 59,000 was estimated. The molecular mass of the native protein was verified by gel filtration on Superose-6 where MAP activity was detected at M_r= 60,000 (Fig. 2*B*). Isoelectric focussing indicated a pI of 5.0 for the protein. Concentrated active MAP preparations were colorless, and in the UV-visible light spectrum no absorbing bands were found above 300 nm. Identical spectra were recorded under aerobic and anaerobic conditions. This indicated that MAP does not contain a specific (redox-sensitive) chromophoric group. MAP was not inactivated after pretreatment with 2 mM EDTA indicating that bivalent cations were not required for protein stabilization.

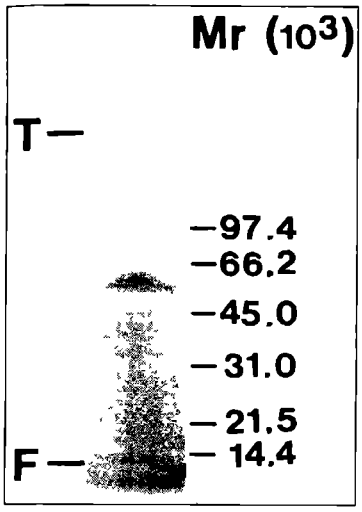

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified Methyltransferase Activation Protein. The protein was prepared for SDS-PAGE as described in "Materials and Methods". A 4-µl sample (2.6 µg protein) was applied to a 10-15% Gradient Phastgel and stained with Coomassie Brilliant Blue. T, top; F, front.

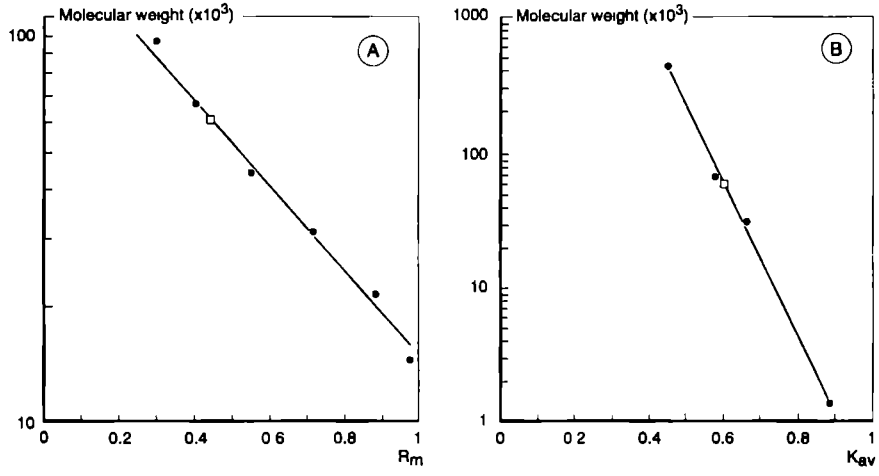

Fig. 2. Estimation of the molecular weight of native and SDS-treated MAP. *(A)*, SDS-treated MAP (□) and markers (●) were applied to a 10-15% Gradient Phastgel. The standards and their molecular weights are given under "Materials and Methods". *(B)*, The native molecular weight of MAP (□) was determined by Superose-6 gel filtration. The column (30 x 1 cm) was developed in 50 mM TES/K^+ (pH 7.0) containing 150 mM NH_4Cl, 15 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM CHAPS, and 10% ethylene glycol at a flow rate of 0.18 ml min^{-1}. The markers (●) and their molecular weights are given in "Materials and Methods". K_{av}, distribution coefficient.

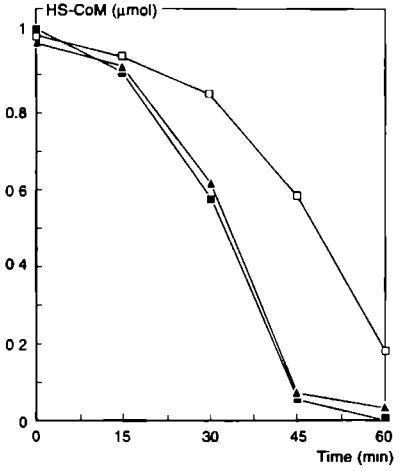

Fig. 3. Effect of 8-azido-ATP and UV illumination on the activity of MAP. Reaction mixtures (final volume, 100 µl) contained 50 mM TES/K^+, 24 mM $MgCl_2$, 10 mM methanol, 10 mM HS-CoM, 2 mM ATP, 1 mM 2-bromoethanesulfonic acid, 20 µl MT_2/hydrogenase fraction, 12.5 µl MT_1 fraction, 10 µl ferredoxin fraction, and 20 µl of treated DEAE-Sepharose fraction of MAP. Incubations were performed under 50% H_2/50% N_2 at 37°C. Assay with MAP after incubation with 8-azido-ATP and UV illumination for 1 hour (–□–). The control experiments contained MAP illuminated in absence of 8-azido-ATP (–■–) and MAP incubated with 8-azido-ATP in the dark (–▲–).

Interaction with 8-azido-ATP. Methyl group transfer of methanol to HS-CoM is strictly dependent on ATP (9). We now studied the effect of 8-azido-ATP on the methyltransferase reaction. The nucleotide is a commonly-used reactive ATP analogue, which can react with all amino acid residues after weak UV illumination and thereby covalently attaches to ATP-binding sites (15). Experiments performed with partly purified MAP obtained from the DEAE-Sepharose column indicated that 8-azido-ATP at least partly could substitute for ATP in the standard assay: if protected from light, activity with 2 mM 8-azido-ATP was about 64% as compared to 2 mM ATP. UV illumination of partly purified MAP in the presence of 8-azido-ATP resulted in a clear retardation of the reaction with respect to MAP controls that had been preincubated in the dark with the ATP analogue or MAP that had been exposed to UV light in the absence of 8-azido-ATP (Fig. 3). These same treatments with either MT_1 or MT_2/hydrogenase fractions had no effect on the methyltransferase activities (data not shown). Since ferredoxin was not absolutely required for methyl group transfer (10),

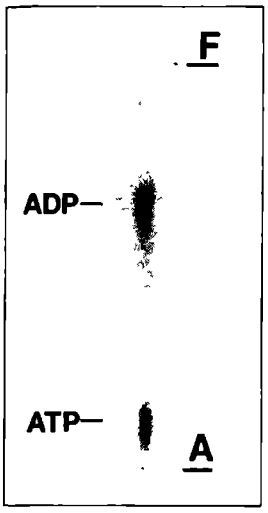

Fig. 4. ATP converting activity of MAP. An autoradiogram of a thin-layer chromatogram used to separate [8-^{14}C]ATP and [8-^{14}C]ADP is shown. The reaction mixture (100 µl) contained 50 mM TES/K^+ buffer (pH 7.0), 2 µM $MgCl_2$, 2 µM [8-^{14}C]ATP, and 40 µl of 79-fold purified MAP (3.06 µg protein) and was incubated for 2 hours under 50% H_2/50%N_2 at 37°C. Sampling and chromatography was as described in "Materials and Methods". A., application line; F, solvent front.

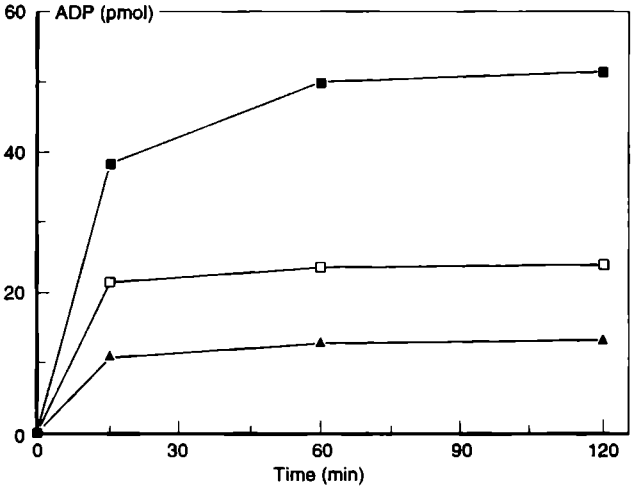

Fig. 5. Time course of ADP production by various amounts of MAP. Reactions were performed as described for Fig. 4, except that the amount of MAP was varied. Addition of 10 µl (–▲–), 20 µl (–□–), and 40 µl (–■–) of MAP corresponds with 12.8, 25.5, and 51.1 pmol of the protein, respectively. Sampling and quantification of [8-^{14}C]ADP was as described under "Materials and Methods".

the electron carrier was not specifically tested for the effect of 8-azido-ATP and UV illumination. The inhibition by 8-azido-ATP and UV-light treatment was confirmed with purified MAP.

ATP conversion. The experiments with 8-azido-ATP indicated that MAP was the site of action of ATP. In agreement herewith, ATP could be converted by the partially purified MAP obtained after DEAE-Sepharose chromatography and the activity coincided with MAP during the subsequent purification steps. Anaerobic incubation of purified MAP with [8-^{14}C]ATP and subsequent thin-layer chromatography and autoradiography of the reaction mixture demonstrated ATP to be converted into ADP (Fig. 4). Time course experiments with varied amounts of MAP showed [8-^{14}C]ADP to be produced at an initial rate of about 0.9 nmol min^{-1} mg protein^{-1} (Fig. 5). However, the reactions stopped when 1.0 ± 0.3 mol [^{14}C]ADP was formed per mol MAP added. Apparently, the reactions came to an end after one catalytic cycle. ATP conversion was not stimulated by higher concentrations of ATP (up to 0.5 mM tested). Nor had the addition of methanol an effect on the rate of hydrolysis or on the final amount of ADP produced. This rules out the possibility that MAP catalyzes the formation of the putative activating substance, methyl-phosphate. The effect of MT_1 on the MAP-catalyzed ATP conversion could not be determined, since the presence of MT_1 interfered with the work-up and subsequent TLC analyses of the reaction mixtures in a way that [^{14}C]ATP and [^{14}C]ADP could not be accurately measured. The ATP-converting activity was remarkably stable. Aerobic incubations of MAP with [^{14}C]ATP did not result in a significant change in ATP conversion. When MAP was kept under air for 3 hours, a 23% decrease in ATP conversion was observed. Only a 12% decrease was obtained, when MAP was heated for 30 min at 100°C. These same treatments caused a complete inhibition of MAP activity in the methyltransferase assay.

Phosphorylation of MAP. Upon incubation of MAP with [8-^{14}C]ATP followed by washing the reaction mixtures on Ultrafree-MC filter units all radioactivity was recovered in the eluate. In contrast, at least part of the activity remained in the protein fraction, when the same experiment was performed with [γ-^{32}P]ATP. Polyacrylamide gel electrophoresis of MAP incubated with [γ-^{32}P]ATP revealed that $^{32}P_i$ was incorporated in the protein fraction in the course of the reaction. The ^{32}P-protein linkage was stable to acid treatment (14). After native 8-25% PAGE clearly labeled bands were observed with apparent molecular masses of 61,000, 120,000, and higher (Fig. 6), indicative of mono-, di- and oligomer forms of ^{32}P-labeled MAP. SDS-PAGE demonstrated the presence of $^{32}P_i$ in a band with a molecular weight of 60,000 and in an indistinct

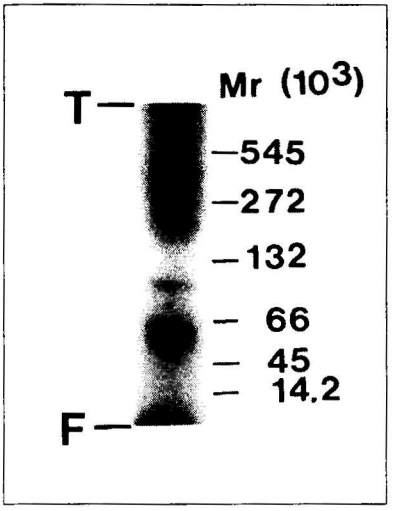

Fig. 6. Phosphorylation of MAP by [γ-^{32}P]ATP. An autoradiogram of a non-denaturing PAGE is shown. Incubation was for 2 hours under air at 37°C. A 4-µl sample (0.13 µg protein) was applied to a 8-25% Gradient Phastgel. The reaction mixture was as described in "Materials and Methods". T, top; F, front.

region around M_r = 27,000 (data not shown). These radioactive bands were also observed after mild treatment with SDS at ambient temperature, which yielded only one 60-kDa band for the nonphosphorylated protein (Fig. 1). This points to an increased instability of MAP-phosphate towards SDS treatment.

DISCUSSION

In *M. barkeri*, transfer of the methyl group of methanol to HS-CoM proceeds via the corrinoid protein MT_1 (7). This protein is only catalytically active when the cobalt atom of its corrinoid prosthetic group is present in the highly reduced Co(I) state (8). Activation of MT_1 required the presence of H_2, hydrogenase, ATP and an as yet not further purified protein fraction called MAP (8,9,10). From the results presented above it is clear that the effect of MAP fraction on the activation of MT_1 was the result of the action of a single protein present in that fraction. MAP was isolated as a protein with a molecular weight of 60,000. The enzyme appeared to be an aerobically unstable and difficult to handle protein with the characteristic property of interacting with other proteins during column chromatography. The purification factor of 79 indicated that MAP comprised 1.3% of the total cell protein.

The inhibitory effect of 8-azido-ATP and UV illumination and the [8-^{14}C]ATP conversion activity proved that MAP was the protein that

interacted with ATP during the activation of MT_1. In fact, MAP was autophosphorylated by the γ-phosphoryl group of ATP. This makes MAP-phosphate the first example of a phosphorylated protein in methanogenic archaea. The autophosphorylating activity of MAP was extremely stable towards high temperature treatment and it is, therefore, conceivable that this activity is perhaps associated with the 27-kDa part of the protein observed after SDS-PAGE of ^{32}P-labeled MAP. The acid stability of the phosphate bond suggests an *O*-phosphate linkage (14).

Both MAP and ATP are absolutely required for activation of MT_1 (10). Since MAP is readily phosphorylated in the presence of ATP and since MAP interacts with ATP during the activation of MT_1 it is evident that phosphorylated MAP is the actual effector in the activation process. For the function of MAP-phosphate 2 modes of action might be envisaged. (i) The phosphate group is transferred to MT_1, affecting the structure of the latter protein and/or its corrinoid prosthetic group in a way that reductive activation is facilitated. In this respect MAP acts like a kinase (16). (ii) Without covalently modifying MT_1, MAP-phosphate alters the structure of the protein and/or its corrinoid prosthetic group. Here, the function of MAP-phosphate resembles the action of chaperone proteins (17). In both mechanisms, the coordination of the 5-hydroxybenzimidazolyl ligand of the corrinoid prosthetic group of MT_1 is eventually modified to an extent that the thermodynamic unfavorable reduction of Co(II) to Co(I) becomes accessible for hydrogen/hydrogenase (18; Chapter 6).

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Activation mechanism of methanol:5-hydroxybenzimidazolylcobamide methyltransferase from *Methanosarcina barkeri*

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Abbreviations used: B_{12}-HBI, 5-hydroxybenzimidazolylcobamide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CH_3-S-CoM, methyl-coenzyme M, 2-(methylthio)ethanesulfonic acid; EPR, electron paramagnetic resonance; HBI, 5-hydroxybenzimidazolyl; HS-CoM, coenzyme M, 2-mercaptoethanesulfonic acid; MAP, methyltransferase activation protein; MT_1, methanol:5-hydroxybenzimidazolylcobamide methyltransferase; MT_2, Co-methyl-5-hydroxybenzimidazolylcobamide:HS-CoM methyltransferase; PAGE, polyacrylamide gel electrophoresis; TES, *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

Activation mechanism of methanol:5-hydroxybenzimidazolylcobamide methyltransferase from *Methanosarcina barkeri*

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SUMMARY

Methanol:5-hydroxybenzimidazolylcobamide methyltransferase (MT_1) is the first of two enzymes responsible for the transmethylation reaction from methanol to 2-mercaptoethanesulfonic acid in *Methanosarcina barkeri*. MT_1 only binds the methyl group of methanol when the cobalt atom of its corrinoid prosthetic groups is present in the highly reduced Co(I) state. Formation of this redox state requires H_2, hydrogenase, methyltransferase activation protein and ATP. Optical and electron paramagnetic resonance spectroscopy studies were employed to determine the oxidation states and coordinating ligands of the corrinoids of MT_1 during the activation process. After isolation MT_1 contained 1.7 mol corrinoids per mol of enzyme with cobalt in the fully oxidized Co(III) state. Water and N-3 of the 5-hydroxybenzimidazolyl base served as the upper and lower ligands, respectively. Reduction to the Co(II) level was accomplished by H_2 and hydrogenase. The cob(II)-amide of MT_1 had the base coordinated at this stage. Subsequent addition of methyltransferase activation protein and ATP resulted in the formation of base-uncoordinated Co(II) MT_1. The activation mechanism is discussed against a new model and compared with those described for other corrinoid-containing methyl group-transferring proteins.

Methanosarcina barkeri can utilize methanol as sole source for methanogenesis and growth. The first step in the reduction of methanol to methane is the formation of an enzyme-bound methylcobamide, catalyzed by methanol:5-hydroxybenzimidazolylcobamide methyltransferase (MT_1) (1). The methyl group of methylated MT_1 is subsequently transferred to 2-mercaptoethanesulfonic acid (coenzyme M,

HS-CoM) by Co-methyl-5-hydroxybenzimidazolylcobamide:HS-CoM methyltransferase (MT_2) (2). As a result methyl-coenzyme M (CH_3-S-CoM) is produced, which is the substrate for the final step in methanogenesis in all methanogens studied so far (3).

The corrinoid prosthetic group of MT_1 can only be methylated by methanol when the central cobalt atom of the cobamide is present in the highly-reduced Co(I) state (4). Since this state is extremely sensitive towards oxidation, MT_1 readily inactivates upon manipulation and even during catalysis. Reactivation is possible and requires participation of a reducing system, Methyltransferase Activation Protein (MAP), and ATP (4,5,6). The reducing system consists of hydrogen, hydrogenase, and ferredoxin. Ferredoxin is not absolutely required, though it stimulates the apparent reaction rate of methyl group transfer (5).

Here, we report the UV-visible absorbance and electron paramagnetic resonance (EPR) properties of the corrinoid prosthetic groups of MT_1 under various additions of the reducing system, MAP, and ATP. From these results the sequence of events leading to the formation of the cob(I)amide of MT_1 is deduced. The activation of MT_1 proceeds by a novel mechanism which is presented in a model and compared with those described for other corrinoid-containing methyl group-transferring proteins.

MATERIALS AND METHODS

Cell material. Cells of *M barkeri* strain MS (DSM 800) were grown and harvested, and cell extract was prepared anaerobically as described before (5,7)

Enzyme assays. Incubation mixtures were prepared in an anaerobic glove box, and reactions were performed in crimp-sealed 10-ml serum vials MT_1 activity was determined by measuring the methanol-dependent HS-CoM conversion to CH_3-S-CoM when added to a reaction mixture containing MT_2/hydrogenase, MAP, and ferredoxin fractions obtained after DEAE-Sepharose fractionation of cell extract of *M. barkeri* (5) A typical reaction mixture (final volume 100 µl) contained 50 mM *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES/K^+) buffer (pH 7 0), 24 mM $MgCl_2$, 10 mM methanol, 10 mM HS-CoM, 2 mM ATP, 1 mM 2-bromo-ethanesulfonic acid, 20 µl MT_2/hydrogenase fraction, 5 µl ferredoxin fraction, 25 µl MAP fraction, and an amount (usually 25 µl) MT_1 to be tested (5) After gassing with 50% H_2/50% N_2 (100 kPa) the vials were kept on ice Reactions were started by placing the vials at 37°C After appropriate incubation periods, by routine 0, 15, 30, 45, and 60 min, reactions were stopped by placing the vials on ice Activity of methyl group transfer of methanol to HS-CoM was routinely assayed by measuring the decrease in the amount of HS-CoM (see below) The methyltransferase activity obtained was linearly dependent on the amount of MT_1 added

The enzymatic activities of MT_2, MAP, hydrogenase and ferredoxin were determined as described previously (4,5,Chapter 5)

Protein purification Because several of the enzymes involved in the methanol HS-CoM methyltransferase reaction are oxygen labile (1,4,5) all handlings were performed in an anaerobic glove box (97 5% N_2/2 5% H_2) at room temperature The purification procedure started by applying 10 ml of cell extract to a DEAE-Sepharose-Cl-6B column and separating the proteins involved in the methyl-transferase reaction as described before (5) MT_2 and hydrogenase activity eluted between 0 20 and 0 22 M NH_4Cl Fractions between 0 25 M and 0 34 M NH_4Cl contained MAP activity MT_1 was present in fractions eluting between 0 39 and 0 42 M NH_4Cl and ferredoxin was obtained between 0 50 and 0 56 M NH_4Cl MT_2/hydrogenase, MAP, and MT_1 fractions were washed by Amicon YM-10 ultra-filtration with 50 mM TES/K^+ buffer (pH 7 0) containing 15 mM $MgCl_2$ and 1 mM dithiothreitol to remove the salt, and were concentrated to a final volume of 3, 6, and 1 ml, respectively Ethylene glycol was added as a stabilizing agent in a final concentration of 10% (vol/vol) Ferredoxin was washed by Amicon YM-3 ultra-filtration and concentrated to a volume of 3 ml Here, ethylene glycol was added in a final concentration of 20%

MT_1 was purified to homogeneity with an anaerobic Perkin-Elmer fast protein liquid chromatography system equipped with a TSK DEAE-5-PW column (7 5 cm by 0 75 cm) After application of 550 µl DEAE Sepharose MT_1 fraction, the column was washed with 15 ml of 50 mM TES/K^+ buffer (pH 7 0) containing 15 mM $MgCl_2$, 1 mM dithiothreitol, and 10% ethylene glycol followed by a 113-ml linear gradient of 0 to 0 25 M NH_4Cl in the buffer Subsequently, the column was washed with 15 ml of 0 5 M NH_4Cl in the buffer to remove all bound proteins The eluate was monitored at 280 nm and fractions of 0 9 ml were collected at a flow rate of 0 45 ml min^{-1} MT_1 activity was present in a large peak that was eluted at about 0 20 M NH_4Cl Fractions eluting between 0 18 and 0 22 M NH_4Cl were pooled This purification step was repeated for the remainder of the DEAE-Sepharose MT_1 fraction The purified MT_1 pools were combined, washed, and concentrated by Amicon YM-10 ultrafiltration to the desired volume, generally 0 4 ml

MAP was purified with a hydroxylapatite column packed with TSK-Gel HA-1000 (7 5 by 0 75 cm) A 1-ml sample was applied to the column and equilibrated with 15 ml of 50 mM TES/K^+ buffer (pH 7 0) containing 1 mM dithiothreitol, 0 5 mM 3-[(3-cholamido-propyl) dimethylammonio]-1-propanesulfonate (CHAPS), and 10% ethylene glycol Bound protein was eluted by a 50-ml simultaneous linear gradient of 50 to 0 mM TES/K^+ and 0 to 300 mM ammonium phosphate buffer (pH 7 0), both buffers contained 1 mM dithiothreitol, 0 5 mM CHAPS, and 10% ethylene glycol Fractions of 0 9 ml were collected at a flow rate of 0 45 ml min^{-1} MAP activity was recovered in a peak that was eluted at 145 mM ammonium phosphate and 26 mM TES The purification step was repeated for the remainder of the DEAE-Sepharose MAP fraction Purified MAP pools were combined, desalted, and concentrated by Amicon YM-10 ultrafiltration by washing with 50 mM TES/K^+ buffer (pH 7 0) containing 1 mM dithiothreitol and 10% ethylene glycol At this stage MAP was purified 23-fold with a recovery of 6 5%, apart from MAP (60 kDa) 8-25% native polyacrylamide gel electrophoresis (PAGE) showed the presence of two contaminating proteins (96 and 32 kDa) that apparently are not related with the methanol HS-CoM methyltransferase reaction (Chapter 5) Since the previously described procedure to purify MAP to homogeneity gave to low yields, the two-step purification method was utilized to purify the activation protein in the large

Table 1. *Purification of methanol 5-hydroxybenzimidazolylcobamide methyltransferase* The purification procedure started from 10 ml of cell extract Enzyme assays were performed as described under Materials and Methods" Units are expressed as μmoles of 2-mercaptoethanesulfonic acid methylated per min

Step	Total protein	Total activity	Specific activity	Factor	Recovery
	mg	*U*	*U/mg*	*-fold*	*%*
Cell extract	260	44 20	0 17	1	100
DEAE-Sepharose	4 4	16 98	3 86	23	38
TSK DEAE	0 6	3 34	5 57	33	8

amounts required for the electron paramagnetic resonance experiments

MT_2/hydrogenase and ferredoxin fractions were each applied to a Sep-pak CM cation exchange cartridge and eluted with 50 mM TES/K^+ buffer (pH 7 0) containing 1 mM dithiothreitol and 10% ethylene glycol to remove fortuitous Mn^{2+} which interfered the EPR experiments

Analytical procedures. UV-visible light absorption spectra were recorded in 1-ml quartz cuvettes on a Hitachi U-3200 spectrophotometer Spectra of MT_1 were recorded against a reference, containing the same components except MT_1, which was replaced by TES/K^+ buffer EPR spectroscopy was carried out on a Bruker 200 D spectrometer equipped with cryogenics, peripheral equipment and data-acquisition/manipulation facilities as described previously (8) Incubation mixtures were anaerobically transferred to EPR tubes under a slight overpressure of hydrogen, and directly frozen in liquid nitrogen EPR spectra of references, containing the same components except MT_1, were also recorded and later subtracted from the spectra of the MT_1 containing samples In the references MT_1 was replaced by TES/K^+ buffer EPR spectra were simulated by using the program KOPER (Hagen, W R , unpublished)

Native PAGE, denaturing SDS-PAGE, and isoelectric focusing were performed with prefabricated minigels using the Pharmacia PhastSystem equipment (Uppsala, Sweden) The gels were stained with Coomassie Brilliant Blue R-250 The subunit molecular weight of MT_1 was determined by electrophoresis on a 10-15% gradient minigel with SDS-buffer strips The markers (Biorad Laboratories, Richmond, CA, USA) were the following (Da) α-lactalbumin (14,400), soybean trypsin inhibitor (21,500), bovine carbonic anhydrase (31,000), hen egg white lysozyme (45,000), bovine serum albumin (66,200), and rabbit muscle phosphorylase *b* (97,400) Native PAGE was performed on a 8-25% gradient minigel with the following markers (Sigma, St Louis, MO, USA) α-lactalbumin (14,200), chicken egg albumin (45,000), the monomer (66,000) and dimer (132,000) of bovine serum albumin, and the trimer (272,000) and hexamer (545,000) of jack bean urease Isoelectric focusing was performed with a pH 3-9 IEF-gel using the pI 3 5-9 6 IEF standard proteins from Biorad (Biorad Laboratories, Richmond, CA, USA)

Protein was determined with the Biorad protein reagent (Biorad Laboratories, Richmond, CA, USA) with bovine serum albumin as a standard Molar concentrations of MT_1 were calculated from the molecular mass (122,000 Da) of the protein (1) Molar amounts of hydroxylapatite-purified MAP were estimated from the

reported M_r = 60,000 of the protein, taking into acount the 23-fold purification factor with respect to 79-fold purified homogenous MAP (Chapter 5) Corrinoids were quantified after conversion into the dicyanocobamide derivatives. Samples of MT_1 were diluted in 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid buffer (pH 10) containing 5 mM potassium cyanide and incubated for 5 min at 90°C (9). Concentrations were calculated from the absorption at 580 nm (ε_{580} = 10.2 x 10^3 M^{-1} cm^{-1}) (10). HS-CoM was measured by the method of Ellman (11) Samples of 25 µl were mixed with 3 ml 0 48 mM 2,2'-dinitro-5,5'-dithiobenzoic acid in 150 mM Tris/Cl buffer (pH 8.0) and immediately measured at 412 nm. Total iron was determined as described by Fish (12) Manganese was measured as described by Bartley and coworkers (13) The effect of bathophenanthroline disulfonate was tested anaerobically essentially as described by Rouviére and Wolfe (14).

Materials. All chemicals used were of analytical grade. HS-CoM, 2-bromoethanesulfonic acid, TES, 3-(cyclohexylamino)-1-propanesulfonic acid, and bathophenanthroline disulfonate were purchased from Sigma Chemical Co (St. Louis, MO.). Dithiothreitol was from Serva Feinbiochemica (Heidelberg, Germany). ATP and CHAPS were purchased from Boehringer (Mannheim, Germany). DEAE-Sepharose-Cl-6B was from Pharmacia LKB Biotechnology A B (Uppsala, Sweden). TSK DEAE-5-PW and TSK-Gel HA-1000 columns were obtained from TosoHaas (Stuttgart, Germany) Sep-pak CM cartridges were acquired from Waters Associates (Milford, MA, USA) Gasses were supplied by Hoek-Loos (Schiedam, The Netherlands) To remove traces of oxygen, H_2-containing gasses were passed over a BASF RO-20 catalyst at room temperature and nitrogen was passed over a prereduced BASF R3-11 catalyst at 150°C The catalysts were a gift of BASF Aktiengesellschaft (Ludwigshafen, Germany)

RESULTS

Purification of MT_1. Methanol:5-hydroxybenzimidazolylcobamide methyltransferase was purified to homogeneity from cell extract of *M. barkeri* strain MS by the simple two-step procedure summarized in Table 1. After this stage only one band, with an estimated M_r = 121.000, was detected upon nondenaturing 8-25% gradient PAGE (Fig. 1). Prior to staining of the gel the protein could be observed as a red colored band due to the presence of corrinoids. Denaturating SDS-PAGE demonstrated the $\alpha_2\beta$ subunit composition of polypeptides of 33,000 and 54,000, respectively, as found before (1). The purification factor of 33 indicated that MT_1 comprised 2.9% of the total cell protein. An average amount of 1.7 ± 0.4 mol 5-hydroxybenzimidazolylcobamide (B_{12}-HBI) per mol of MT_1 was found from three separate purifications. Treatment of MT_1 with 50 mM EDTA, which removed all Mg^{2+}, and subsequent native PAGE revealed the loss of the corrinoid prosthetic group and the break-up of the protein into its subunits. Apparently, Mg^{2+} plays a role in binding the corrinoid prosthetic group and in

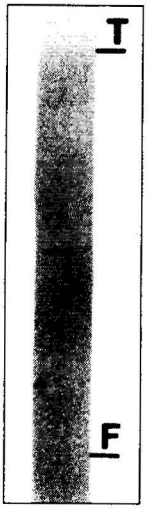

Fig. 1. Polyacrylamide gel electrophoresis of purified methanol:5-hydroxybenzimidazolyl-cobamide methyltransferase (MT_1). A 4-µl sample (2.0 µg protein) was applied to a 8-25% Gradient Phastgel and stained with Coomassie Brilliant Blue. T, top; F, front.

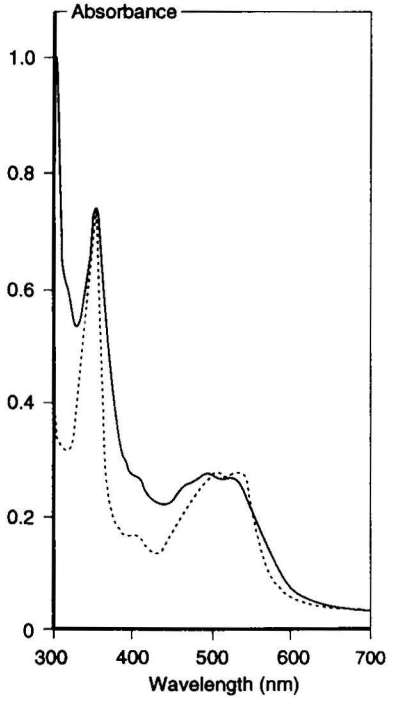

Fig. 2. Absorption spectrum of MT_1 as-isolated. The spectrum of MT_1 (0.82 ml; 2.51 mg/ml; 20.6 µM) was recorded in an anaerobic 1-ml quartz cuvette at 37°C under 50% H_2/50% N_2 in 50 mM TES/K^+, pH 7.0, containing 15 mM $MgCl_2$, 1 mM dithiothreitol, and 10% ethylene glycol (——). The spectrum of non-protein bound Co-aquo-5-hydroxybenzimidazolylcobamide (aquo-B_{12}-HBI) (26 µM) was recorded in the same buffer (- - - -).

subunit association. From isoelectric focusing a pI of 4.5 was obtained. Determination of non-heme iron revealed the presence of 1.7 mol Fe/mol MT_1. Pretreatment with 2 mM of the iron-chelator bathophenanthroline disulfonate did, however, not result in any loss of activity. In addition, EPR spectroscopical studies of MT_1 at 14 K, in absence and presence of 2 mM dithionite, did not reveal any signals indicative of the presence of iron-sulfur clusters. From this, it was concluded that MT_1 does not contain functional active iron.

UV-visible spectroscopy. MT_1 was anaerobically isolated as a red colored protein and the UV-visible spectrum of the prosthetic group (Fig. 2) was typical for Co(III) corrinoids (15,16,17). No change in the spectrum occurred upon aerating the sample. The prosthetic group showed absorption maxima at 352 (2.89), 493 (1.07), and 522 (1.04) nm with shoulders at 388 (1.16), 403 (1.04), and 465 (1.0) nm (the numbers in parentheses express the absorbance relative to that of the shoulder at 465 nm). The major visible absorption band at 352 nm is characteristic of the presence of water as the upper ligand (16,17). Comparison of the spectrum of aquo-B_{12}-HBI with that of the corrinoid of MT_1 showed that the absorbance of the prosthetic group in the 375 to 475 nm region was relatively increased.

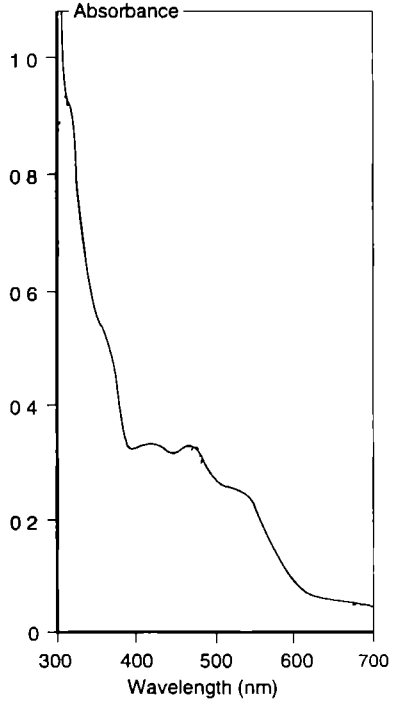

Fig. 3. Absorption spectrum of hydrogen-reduced MT_1 Spectra were recorded as described under Fig 2 Absorption spectrum of MT_1 (0 82 ml, 2 51 mg/ml; 20 6 µM) after incubation for 90 min at 37°C in the presence of 12 µl MT_2/hydrogenase and 8 µl ferredoxin fraction (——) The spectrum of the cob(II)amide of B_{12}-HBI (33 µM) was recorded in an identical way (- - - -)

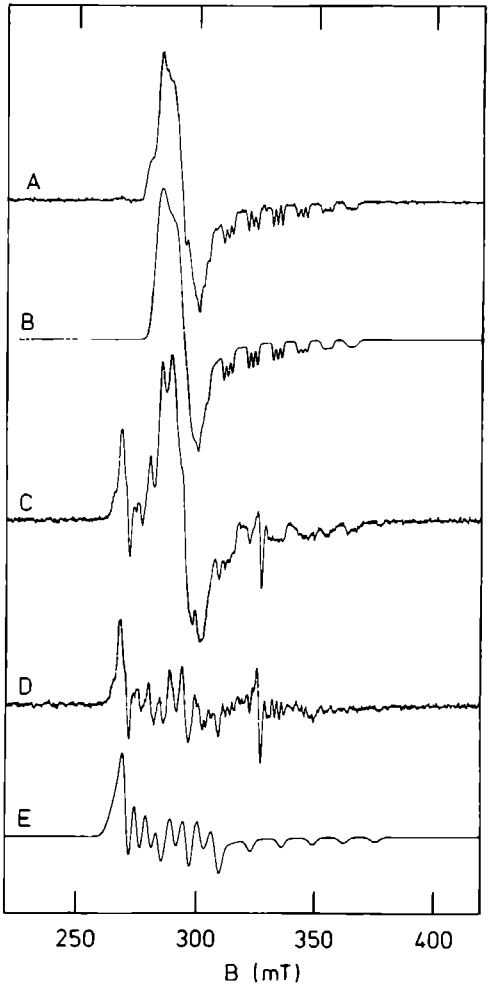

Fig. 4. Low-spin Co(II) EPR spectra of MT_1. The corrinoids of MT_1 were reduced to the Co(II) level with hydrogenase and ferredoxin as described under Fig. 2 followed by storage for 16 h at 4°C. Trace *A*: 2x averaged spectrum of the "base-on" form of Co(II) MT_1 (35 mg/ml; 287 μM). Trace *B*: simulation of *A*. Trace *C*: 9x averaged, baseline corrected spectrum of Co(II) MT_1 (1.93 mg/ml; 15.8 μM) obtained after incubation with MAP (0.45 mg/ml; 7.5 μM) and ATP (5 mM) for 90 min at 37°C under 5% H_2/ 95% N_2; the spectrum shows that part of the MT_1 is still in the "base-on" form. Trace *D*: difference spectrum of *C* minus *A*. Trace *E*: simulation of *D*. See the Results section and Table 2 for details on the simulations. EPR conditions: microwave frequency, 9.18 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.63 (trace *A*) and 0.8 mT (trace *C*); microwave power, 0.08 (trace *A*) and 0.32 mW (trace *C*); temperature, 24 (trace *A*) and 19 K (trace *C*).

Incubation of MT_1 with a small amount of MT_2/hydrogenase and ferredoxin fraction under hydrogen resulted in a change in color of the corrinoid protein. The reaction mixture turned yellow and displayed the typical spectrum (Fig. 3) of the Co(II) form of the cobamide (15,16,18). Absorption maxima were observed at 417 (1.31) and 465 (1.29) nm with shoulders at 315 (3.64), 356 (2.11), and 535 (1.0) nm (the numbers in parentheses indicate the absorbance relative to that of the shoulder at 535 nm). The Co(II) prosthetic group of MT_1 exhibited a relative increase in absorbance around 400 nm and in the 500 to 600 nm region compared to the spectrum of free Co(II) B_{12}-HBI (16). Prolonged incubation or the addition of increased amounts of hydrogenase and ferredoxin did not result in any alterations of the spectrum. Ferredoxin was not absolutely required for this reduction. Addition of MAP, up to an equimolar concentration of MT_1 tested (20.6 μM), or addition of ATP, up to a concentration of 5 mM tested, did not alter the Co(II) spectrum. However, when both MAP and ATP were added the absorbance at 315 nm of the cob(II)amide of MT_1 was distorted (not shown). No significant changes occurred at higher wavelengths. Since such a distortion was also observed in the UV-visible spectrum of free Co(II) B_{12}-HBI when coordination of the 5-hydroxybenzimidazolyl (HBI) nucleotide was affected (16), we investigated this effect further by EPR spectroscopy.

EPR spectroscopy. EPR observations of corrinoids are limited to the paramagnetic Co(II) state of cobalt. When MT_1 was studied in the as-isolated state no signal was detected. After incubation of MT_1 with hydrogenase and ferredoxin under hydrogen the typical signal of the nucleotide base-coordinated ("base-on") form of Co(II) corrinoids (Fig. 4*A*) was observed (19). The spectrum could be simulated on the basis of a near-axial, low-spin Co(II) complex with hyperfine splitting from the I=7/2 cobalt nucleus and superhyperfine splitting from an axially coordinating nitrogen with I=1 (Table 2 and Fig. 4*B*). For a reasonable fit it was required that the line width in the z-direction was a function of the cobalt nuclear quantum number m_I. In the perpendicular (or xy-) direction there was no resolution. Therefore, the combined xy-values for g, A, and W were not uniquely determined. However, it was found by extensive fitting that the low-field shoulder to the experimental spectrum could not be simulated by any combination of these parameters. This shoulder might well be a manifestation of non-collinearity of the g- and A(Co)-tensors, i.e., the actual symmetry of the coordination site was probably triclinic.

In the presence of MAP and ATP the EPR spectrum of Co(II) MT_1 changed (Fig. 4*C*). The spectrum was a sum of two Co(II) spectra: a

Table 2. *Simulation parameters for the low-spin Co(II) EPR spectra of MT_1.* Powder patterns were generated from 201 x 11 molecular orientations. The spin Hamiltonian was $H = \beta BgS + SA_{Co}I + SA_NI$. W_o is the width of a Gaussian in magnetic field space, and the line width was a function of the central nuclear quantum number m_I according to $W = W_o + Bm_I + C(m_I)^2$. All tensors g, A_{Co}, A_N, W_o, B, and C are taken to be collinear.

Tensor		Orientation		
		(*x*)	(*y*)	(*z*)
Base-on form:				
g		2.26	2.22	2.01
A_{Co}	(mT)	0.5	0.5	10.4
A_N	(mT)	0	0	1.8
W_o	(mT)	3.2	3.2	1.6
B		0	0	0.03
C		0	0	0.05
Base-off form:				
g		2.32	2.29	2.00
A_{Co}	(mT)	5.0	5.3	13.0
W_o	(mT)	4.5	2.0	1.7
B		0	0	0.1
C		0	0	0

"base-on" and a base-uncoordinated ("base-off") spectrum. The cobalt hyperfine splitting of the latter could be seen to extend to higher field values than that of the former. This pattern became somewhat obscured when we subtracted the pure "base-on" spectrum from the sumspectrum (resulting in trace *D* in Fig. 4). Because of the low signal-to-noise ratio, however, the xy-pattern of the "base-off" spectrum was now more easily identified, thus allowing for a simulation of this spectrum (Table 2 and Fig. 4*E*). The formation of "base-off" Co(II) MT_1 indicated that, as the result of the action of MAP and ATP, the HBI-base was no longer coordinated and that no ligand or a weak ligand with poor delocalization properties was present (19).

DISCUSSION

Optical and EPR spectroscopical studies were employed to determine the oxidation state of the central cobalt atom and the coordination of the ligands in the corrinoid protein MT_1 under various additions of MAP, ATP, and a reducing system. After isolation MT_1 contained 1.7 mol B_{12}-HBI per mol protein. The UV-visible light spectrum (Fig. 2) indicated that the prosthetic group was present in the hexa-coordinated

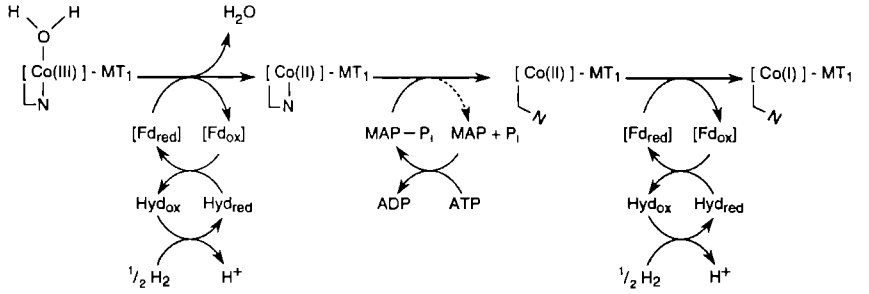

Fig. 5. Proposed scheme for the reductive activation of MT_1. [Co(III)], [Co(II)], and [Co(I)] represent the various reduction states of the cobalt atom of the corrinoids of MT_1. Coordination of N-3 of the 5-hydroxybenzimidazolyl base is illustrated by the connecting line between Co and N. In the "base-off" Co(II) state no ligand is shown but it is possible that a ligand with poor delocalization properties, e.g. water, is present. The dashed line indicates that it is not fully known how MAP-phosphate is dephosphorylated. Eventually, MT_1 with cobalt in the Co(I) state is produced which is able to bind the methyl group of methanol. Hyd, hydrogenase; Fd, ferredoxin. Ferredoxin is shown in parenthesis because activation can occur in absence of this protein (5).

Co(III) oxidation state with the nucleotide 5-hydroxybenzimidazole and water as the lower and upper ligands, respectively. As isolated MT_1 is inactive and reactivation requires the reduction of Co(III) to Co(I), which is brought about by a reducing system (H_2, hydrogenase, and ferredoxin), MAP, and ATP (4,5,Chapter 5). Incubation of MT_1 with hydrogen, hydrogenase, and ferredoxin resulted in the reduction to the Co(II) state. EPR spectroscopy demonstrated that the HBI-base was still coordinated at this stage ("base-on"). Addition of both MAP and ATP induced a conversion of "base-on" into "base-off" Co(II) MT_1. In non-protein bound B_{12}-HBI such "base-off" conversion increases the midpoint redox potential of the Co(II)/Co(I) from -592 to -500 mV (16). In a similar way, the action of MAP and ATP may facilitate the Co(II) to Co(I) reduction of MT_1. Since in our previous paper (Chapter 5) we showed MAP to be autophosphorylated by ATP, we suggest that it is MAP-phosphate which interacts with MT_1 in a way that the HBI-base becomes dissociated. The proposed sequence of events in the reductive activation of MT_1 is summarized in Fig. 5. The final step in the scheme is identical to the ATP-independent reductive activation of the corrinoid/iron-sulfur proteins involved in acetyl-CoA synthesis and degradation that have been isolated from *Clostridium thermoaceticum* (20) and from *Methanosarcina thermophila* (21). These proteins were

purified in the inactive cob(II)amide form at which the corrinoids within their protein environment already were contained in the - at neutral pH - thermodynamic unfavorable "base-off" state. Reduction to the catalytically active Co(I) state, which physiologically has to be performed by electrons derived from the carbonyl (CO)/CO_2 oxidation (E_0' = -520 mV), occurred at midpoint redox potentials of -504 mV (22) and -486 mV (21). Note that the latter values about equal the E_0' = -500 mV of the free "base-off" cob(II)amide/cob(I)amide couple (16).

In comparing the UV-visible light spectra of MT_1 with an aqueous solution of B_{12}-HBI we noticed some differences in the 400 nm and 500-600 nm regions (Figs. 2 and 3). Since MT_1 does not contain Fe-S clusters (this paper) or other chromophoric groups (results not shown), this had to be caused by a conformational distortion of the corrin ring structure by the protein (23). Such distortion is likely to change the reduction potential of the prosthetic group. Indeed, for a number of corrinoid-containing methyltransferases midpoint potentials have been measured that were significantly higher than found for the corresponding B_{12} derivatives in solution (21,22,24). With respect to the membrane-bound methyltetrahydromethanopterin:HS-CoM methyltransferase complex from *Methanosarcina mazei* an E_0' as high as -426 to -450 mV was established for the reduction of "base-on" cob(II)amide to cob(I)amide, which is about 150 mV more positive than for free B_{12}-HBI (25). Incubation with ATP and the methyl donor (methyltetrahydromethanopterin) caused the apparent midpoint potential to raise another 200 mV (E_0' = -245 mV), i.e. to a level that reduction becomes feasible at even very low hydrogen concentrations (E_0' = -414 mV). Remarkably, the 200 mV shift was not observed when the methyl donor was omitted and the authors (25) propose that the simultaneous action of ATP and the methylating substrate in a ternary enzyme complex is required for raising the redox potential. In agreement herewith, we never observed the characteristic UV-visible light spectral features of cob(I)amide, when MT_1 was incubated with MAP and ATP under high (100 kPa) hydrogen partial pressure. However, the additional presence of methanol at even low concentrations (<100 μM) resulted in the instantaneous formation of methyl-B_{12}-HBI in MT_1 (5). Future investigations must focus on questions with respect to the corrinoid midpoint redox potentials in MT_1 and the effects hereon of MAP, ATP, and methanol.

To be active corrinoid-dependent methyltransferases often require an ATP-dependent reductive activation (3,26). As yet, only the activation mechanism of methionine synthase has been elucidated (27). Here, ATP is the substrate in the formation of the potent methylating agent,

S-adenosyl-methionine, which traps Co(I) out of the thermodynamic unfavorable Co(II) to Co(I) reduction equilibrium (24). In this and in our previous paper (Chapter 5) we have presented evidence that nature developed another approach to facilitate the generation of cob(I)amide, notably by inducing in an ATP-dependent process the conformational change of the prosthetic group. Perhaps, other corrinoid-containing methyltransferases from methanogens (3) and other obligate anaerobic organisms (28) are activated in a similar fashion.

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Methyl-tetrahydromethanopterin synthesis from methanol, the first step in methanol oxidation in *Methanosarcina barkeri*

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Abbreviations used: B_{12}-HBI, 5-hydroxybenzimidazolylcobamide; CH_3-S-CoM, methyl-coenzyme M, 2-(methylthio)ethanesulfonic acid; CoM-S-S-HTP, the heterodisulfide of HS-CoM and HS-HTP; HS-CoM, coenzyme M, 2-mercaptoethanesulfonic acid; H_4MPT, 5,6,7,8-tetrahydromethanopterin; H_4SPT, 5,6,7,8-tetrahydrosarcinapterin, a H_4MPT derivative with an additional glutamyl group; HS-HTP, 7-mercaptoheptanoylthreonine phosphate; MT_1,methanol:5-hydroxybenzimidazolylcobamide methyltransferase; MT_2, Co-methyl-5-hydroxybenzimidazolylcobamide: HS-CoM methyltransferase.

Methyl-tetrahydromethanopterin synthesis from methanol, the first step in methanol oxidation in *Methanosarcina barkeri*

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SUMMARY

Cell-free extract of *Methanosarcina barkeri* was able to convert methanol into methane and CO_2 under N_2 5-Methyl-tetrahydromethanopterin was the first intermediate observed in the oxidation route of methanol Synthesis of this compound from methanol and tetrahydromethanopterin (H_4MPT) was specifically catalyzed by the membrane fraction Ti(III)citrate and incubation under H_2 stimulated the reaction ATP could substitute for Ti(III)citrate Since, the membrane fraction did not contain methanol 5-hydroxybenzimidazolylcobamide methyltransferase or 5,10-methylenetetrahydromethanopterin reductase activity, 5-methyl-H_4MPT synthesis had to proceed by a direct transfer of the methyl group of methanol to H_4MPT In absence of H_4MPT a small amount of cobamide was methylated by the methyl group of methanol, suggesting the involvement of a corrinoid-containing methyltransferase

Methanosarcina barkeri is one of the most versatile methanogenic archaea in its ability to grow on H_2/CO_2, methanol, methylamines, and acetate (1) In the presence of H_2, methanol is reduced to methane but acetate must be supplemented to ensure growth (2) Reduction of methanol occurs via methyl-coenzyme M (CH_3-S-CoM), the substrate of the methane-forming reaction Synthesis of CH_3 S-CoM from methanol and coenzyme M (HS-CoM) is catalyzed by the concerted action of two methyltransferases (3) First, the methyl group of methanol is bound to the corrinoid prosthetic group of methanol 5-hydroxybenzimidazolylcobamide methyltransferase (MT_1) (4) The methyl group of methylated MT_1 is subsequently transferred to HS-CoM by Co-methyl-5-hydroxybenzimidazolylcobamide HS-CoM methyltransferase (MT_2) (3)

When methanol is used as the sole substrate for growth, part of the methanol has to be oxidized to generate the reducing equivalents for the

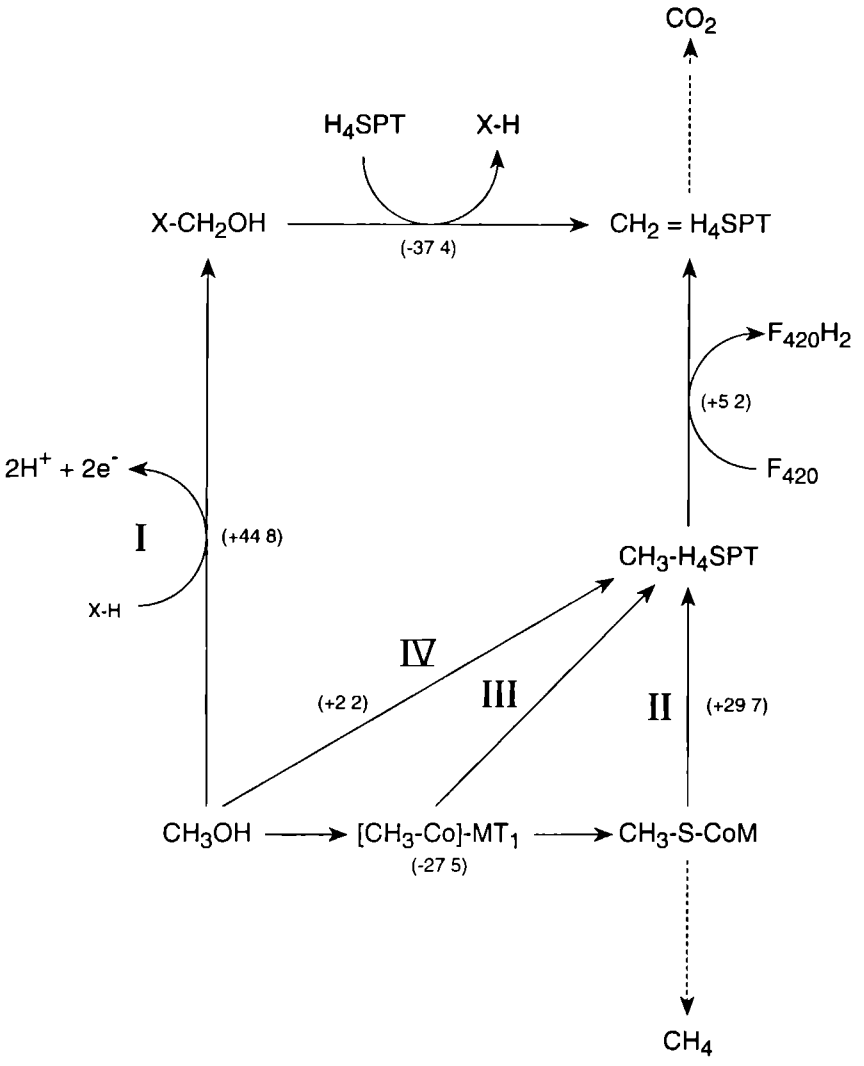

Fig. 1. Alternative pathways for the methanol methyl group oxidation Roman numbers next to arrows indicate the routes as proposed in the introduction The numbers in parentheses represent free energy changes in kJ/mol of reactions in the indicated direction under standard conditions F_{420}, a 8-hydroxy-5-deazaflavin derivative, X, unknown one-carbon carrier suggested by Blaut and Gottschalk (8)

CH_3-S-CoM reduction to methane (Eqn. 1).

$$4CH_3OH \longrightarrow 3CH_4 + CO_2 + 2H_2O \qquad (1)$$

The presence in high concentrations of the relevant enzymes suggests that methanol oxidation largely follows the same metabolic route as CO_2 reduction to methane, though in the reversed order (5,6). In addition, from experiments with whole cells of *M. barkeri* and *Methanosarcina mazei* strain Gö1 it could be concluded that the oxidation of methanol to the formaldehyde level must be dependent on a sodium-motive force (7). However, the initial step in the oxidation route of methanol is still not clear (5,7). On the basis of the literature several pathways are conceivable (Fig. 1). (I) Methanol oxidation could proceed by use of a methanol dehydrogenase and an unknown one-carbon carrier X (8). Here, sodium-motivated reversed electron transport should drive the strongly endergonic oxidation of methanol to the formaldehyde level ($\Delta G^{\circ'}$= +44.8 kJ/mol) (8). In this case methanol would enter the oxidation pathway at the level of methylene-tetrahydrosarcinapterin (methylene-H_4SPT). The presence, however, of methanol dehydrogenase has never been demonstrated. (II) Methanol could be converted to CH_3-S-CoM by the MT_1/MT_2 system mentioned above, followed by the methyl group transfer to H_4SPT in a reaction catalyzed by the membrane-bound, sodium-translocating methyl-H_4SPT:HS-CoM methyltransferase (9,10). In this direction, the reaction is highly endergonic ($\Delta G^{\circ'}$= +29.7 kJ/mol) (1) and could be driven by sodium import. (III) The methylated corrinoid protein MT_1 is the substrate in the transfer of the methyl group of methanol to H_4SPT. (IV) Methyl-H_4SPT is formed directly from methanol and H_4SPT. Under standard conditions, this reaction is estimated to be only slightly endergonic ($\Delta G^{\circ'}$= +2.2 kJ/mol) (5).

Here, we demonstrate that cell-free extract of *M. barkeri* is able to synthesize methyl-H_4MPT from methanol and H_4MPT. The reaction was specifically catalyzed by a membrane fraction which did not contain MT_1 or 5,10-methylene-tetrahydromethanopterin reductase. Therefore, we suggest a direct transfer of the methyl group of methanol to H_4MPT.

MATERIALS AND METHODS

Organisms and extracts. *M. barkeri* strain MS (DSM 800) and *M. thermoautotrophicum* strain ΔH (DSM 1053) were grown in a 300-l fermentor on mineral

medium under 80% N_2/20% CO_2 with 250 mM methanol (16), and on synthetic medium under an 80% H_2/20% CO_2 atmosphere (17), respectively Cells were harvested at the end of exponential growth and stored at -70°C under N_2 until use Cell-free extract of *M barkeri* was prepared by passage through a French pressure cell as described before (18) Coenzyme depleted extract was prepared under anaerobic conditions by extensive washing the cell extract on a PM-30 (Amicon) membrane with 50 mM TES buffer containing 15 mM $MgCl_2$ and 1 mM dithiothreitol Boiled cell-free extracts for H_4MPT purification were prepared from cells of *M thermoautotrophicum* as described previously (14) Protein was determined with the Coomassie Brilliant Blue G-250 method (19) using bovine serum albumin as a standard

Enzyme assays. Incubation mixtures were prepared inside an anaerobic glove box and reactions were performed in crimp sealed 10 ml serum vials Transfer of the methyl group of methanol to H_4MPT was determined in reaction mixtures (200 µl) containing 50 mM TES buffer pH 7 0, 25 mM $MgCl_2$, 50 mM methanol, 0 11 mM H_4MPT, 5 mM Ti(III)citrate, 0 5 mM 2-bromoethanesulfonate, and 100 µl protein fraction After gassing with H_2 or N_2 (100 kPa) the vials were kept on ice Reactions were started by placing the vials at 37°C After appropriate incubation periods reactions were stopped by placing the vials on ice Methyl-H_4MPT synthesis was determined by anaerobic HPLC analysis of the incubation mixtures

Reaction mixtures for methanol conversion to methane (final volume, 200 µl) contained 100 mM TES buffer pH 7 0, 24 mM $MgCl_2$, 45 mM methanol, 2 4 mM ATP, and 100 to 170 µl cell free extract (24 mg protein/ml) After gassing with the appropriate gas or gas mixture 100 µl ethane was added as an internal standard Reactions were started by placing the vials at 37°C After each time interval 0 3 ml gas samples were drawn for analyses and methane was measured on a Poropack Q (80/100) column as described by Hutten et al (16) Methanol was measured with 4 8 mM 1-propanol as an internal standard on a Hewlett Packard 5890A gas chromatograph according to the method of Teunissen et al (20) Reaction mixtures for formaldehyde conversion to methane were essentially the same except that 5 mM formaldehyde was added instead of methanol Reduction of CH_3-S-CoM to methane was determined with reaction mixtures similar to those described for methanol reduction except that 10 mM CH_3-S CoM was added instead of methanol Conversion of formaldehyde to CH_3 S-CoM in the presence of 2-bromoethanesulfonate (0 5 mM) was assayed as described by Poirot et al (21) CH_3-S-CoM synthesis from methanol and HS-CoM in the presence of 50 µl cell extract (1 2 mg protein) and 2-bromoethanesulfonate (0 5 mM) was assayed as described previously (18) Isotachophoresis was used for the determination of CH_3-S CoM (22) Reduction of CoM-S-S-HTP was measured by following the rate of thiol formation with 2,2'-dinitro-5,5'-dithiobenzoic acid (23) essentially as described by Hedderich and Thauer (24) Activities were determined by incubating a series of anaerobically prepared reaction mixtures (200 µl) in crimp-sealed 10-ml serum vials containing 1 64 mM CoM-S-S-HTP and cell extract (60 µg to 4 mg protein, depending on the activities) in 100 mM TES buffer pH 7 0 at 37°C for appropriate periods of time Hydrogen (100 kPa), formaldehyde (5 mM), or methanol (25 mM) served as the reactants, with formaldehyde and methanol incubations took place under nitrogen (100 kPa) in the presence of 0 5 mM 2-bromoethanesulfonate Thiol concentrations (HS-CoM, HS-HTP) were assayed spectrophotometrically according to Ellman (23)

5,10-Methylene-tetrahydromethanopterin reductase activity was determined as described by te Brommelstroet et al (6) and methanol HS CoM methyltransferase activity was assayed as reported by Daas et al (18)

Fractionation of methanol:H_4MPT methyltransferase activity. All handlings were performed in an anaerobic glove box (97 5% N_2/2 5% H_2) at room temperature The procedure started by separating PM-30 washed extract (3,5 ml, 84 mg protein) on a column packed with DEAE-Sepharose Cl-6B (12 by 2 8 cm) equilibrated in 20 mM potassium phosphate buffer pH 7 Elution with 100 ml of equilibration buffer yielded the pass-through fraction Bound proteins were eluted in a single step with 100 ml 0 6 M NH_4Cl in the phosphate buffer The eluate was monitored at 280 nm and 3 5-ml fractions were collected at a flow rate of 2 ml/min The fractions eluting with NH_4Cl were combined, washed by Amicon YM-10 ultrafiltration with 20 mM potassium phosphate buffer pH 7 to remove the salt, and concentrated to a final volume of 3 5 ml The opaque pass through fractions were combined (10 5 ml) and anaerobically ultracentrifuged in 3 5-ml polyallomer tubes for 18 h at 100,000 x *g* and 4°C The supernatant was carefully decanted and the pellet was resuspended in 0 5 ml 20 mM potassium phosphate buffer pH 7 All methanol H_4MPT methyltransferase activity was present in the pellet fraction

Chromatographic analysis. Methanopterin derivatives were analyzed and quantified by HPLC taking care of strict anoxic conditions during sample preparation and subsequent analysis Reaction mixtures were boiled for 3 min and denatured proteins were pelleted by centrifugation for 10 min at 16,000 x *g* The supernatants obtained were filtered over a 0 45 µM luer-lock filter (Millipore, Bedford, MA, USA) and 50 µl samples were subjected to reversed-phase HPLC analysis on a Hewlett-Packard 1048B HPLC, equipped with a Hewlett-Packard 1040A diode-array detector coupled to a Hewlett Packard 85B computing integrator HPLC was performed at 35°C on a 10 µm LiChrosorb RP-18 column (250 by 4 mm, Alltech Europe, Eke, Belgium) Separation was achieved by applying a 10 min linear gradient of 5% to 25% methanol in 40 mM sodium formate buffer pH 3, followed by 15 min of isocratic elution with 25% methanol in the buffer, the flow rate was 0 8 ml/min The eluate was monitored at 260 nm When a peak was recognized as such, complete UV-visible light spectra (230-400 nm) were recorded continuously Peaks were identified by their retention time and UV-visible light spectra The retention times of H_4MPT and methyl-H_4MPT were 17 7 and 20 2 min, respectively The extinction coefficients for H_4MPT and methyl-H_4MPT at 260 nm and pH 3 were determined to be 14 1 and 5 7 mM^{-1} cm^{-1}, respectively Before use, the HPLC buffer solutions were sparged at 40°C with helium gas for at least 1 h, and the column was eluted for 18 h with 25% methanol in the buffer at a flow rate of 0 1 ml/min Sparging of the solutions at 40°C was continued throughout the subsequent HPLC runs

Reaction mixtures for corrinoid analysis were essentially the same as described for the methanol H_4MPT methyltransferase assay, except that no H_4MPT was added and the volume of the reaction mixtures was increased to 6 ml Because of the light sensitivity of methylated corrinoids the vials were wrapped in aluminum foil and all handlings were carried out in the dim light of a red lamp Incubation was for 60 min at 37°C under H_2-atmosphere Extraction and HPLC analysis were performed as described before (18)

Table 1. *Specific activities of various reactions catalyzed by cell-free extract of Methanosarcina barkeri* Reactions were performed as described in "Materials and Methods" Reaction rates were determined by following the substitution, conversion, or production of the compounds printed in italics

Reactions studied	Specific activities (mU/mg protein)[a]
$4CH_3OH \longrightarrow 3\mathit{CH_4} + CO_2$	0 8 (2 7)[b]
$CH_3OH + H_2 \longrightarrow \mathit{CH_4} + H_2O$	29 (39)
$CH_3OH + \mathit{HS}\text{-CoM} \longrightarrow CH_3\text{ S CoM} + H_2O$	44
$CH_3\text{-S-CoM} + H_2 \longrightarrow \mathit{CH_4} + \text{HS-CoM}$	24
$HCHO + 2H_2 \longrightarrow \mathit{CH_4} + H_2O$	32
$2HCHO \longrightarrow \mathit{CH_4} + CO_2$	32
$HCHO + \mathit{HS}\text{-CoM} + H_2 \longrightarrow CH_3\text{-S-CoM} + H_2O$	28
$\text{CoM-S-S-HTP} + H_2 \longrightarrow \mathit{HS}\text{-CoM} + \mathit{HS}\text{-HTP}$	140
$2\text{CoM-S-S-HTP} + HCHO \longrightarrow 2\mathit{HS}\text{-CoM} + 2\mathit{HS}\text{-HTP} + CO_2 + H_2O$	14
$3\text{CoM-S-S-HTP} + CH_3OH \longrightarrow 3\mathit{HS}\text{-CoM} + 3\mathit{HS}\text{-HTP} + CO_2$	0 6

[a] One milliUnit (mU) is defined as 1 nmol of substrate converted per min

[b] The numbers in parentheses represent the specific activity of substrate conversion in the presence of 2 2 mM CoM-S-S HTP

When [^{14}C]methylated corrinoids were extracted reaction mixtures were the same as described above, except that 10 mM [^{14}C]methanol (0 13 TBq/mol) was used, the volume of the reaction mixtures was 400 µl Incubation and extraction were performed as mentioned before (18), except that the residue obtained was dissolved in 100 µl of 80% ethanol Aliquots of 30 µl were spotted on 0 2 mm DC Plastikfolien Kieselgel 60 TLC plates from Merck and developed with methanol/acetic acid/water (7/1/12, vol/vol) The R_f value of authentic methyl-B_{12}-HBI was 0 47 Radioactivity was located by autoradiography for 8 days at -80°C using Kodak XAR-5 X-ray film

Materials. All chemicals were of analytical grade HS-CoM, TES, and 2-bromoethanesulfonate were purchased from Sigma Chemical Co (St Louis, MO, USA) Formaldehyde and Ti(III)chloride were from Merck-Schuchardt A G (Darmstadt, Germany) Dithiotreitol was obtained from Serva Feinbiochemica (Heidelberg, Germany) ATP was from Boehringer GmbH (Mannheim, Germany) Methanol (HPLC-grade) was obtained from J T Baker (Deventer, The Netherlands) [^{14}C]Methanol (0 13 TBq/mol) came from New England Nuclear (Boston, MA, USA) DEAE-Sepharose-Cl-6B was from Pharmacia LKB Biotechnology A B (Uppsala, Sweden) Ti(III)citrate was prepared from Ti(III)chloride and sodium citrate according to Zehnder and Wuhrmann (11) CH_3-S-CoM was prepared by methylation of HS-CoM with dimethylsulfonic acid as described before (12) HS-HTP and CoM-S-S-HTP were synthesized according to Ellermann et al (13) H_4MPT, from *Methanobacterium thermoautotrophicum* strain ΔH, and 5-methyl-H_4MPT were isolated and prepared as described by te Brommelstroet et al (14) Methyl-B_{12}-HBI was synthesized as described by Pol et al (15) Gasses were

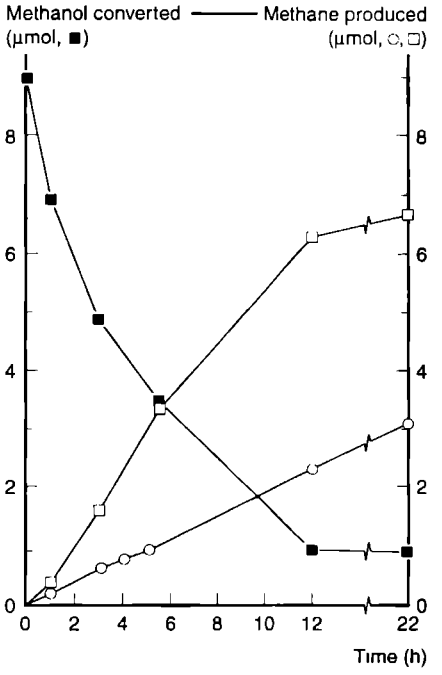

Fig. 2. Methane production and methanol consumption under N_2 by cell-free extract of *Methanosarcina barkeri*. The reactions were performed as described under "Materials and Methods" in the presence of 4 mg protein and 10 mM HS-CoM. Methane production (—□—) and methanol conversion (—■—) with CoM-S-S-HTP (2.2 mM) added. For comparison, methane production of a reaction mixture in absence of CoM-S-S-HTP is plotted (—○—).

supplied by Hoek-Loos (Schiedam, The Netherlands). To remove traces of oxygen, H_2-containing gasses were passed over a BASF RO-20 catalyst at room temperature and N_2 was passed over a prereduced BASF R3-11 catalyst at 150°C. The catalysts were a gift of BASF Aktiengesellschaft (Ludwigshafen, Germany).

RESULTS

Methanol conversion by cell-free extract of *M. barkeri*. Cell-free extracts of *M. barkeri* strain MS were able to produce methane from methanol when incubated under N_2 atmosphere (Fig. 2). Specific activities were, however, quite low: 0.8 nmol/min · mg protein; which was 30-fold lower compared to methanogenesis from methanol and H_2 (Table 1). The activity was increased to 2.7 nmol/min · mg protein, when CoM-S-S-HTP (2.2 mM) was included in the assays (Fig. 2, Table 1). Both under N_2 and H_2 atmosphere no activity was found in absence of ATP. The effect of ATP and CoM-S-S-HTP indicates that the methanogenic activity can be attributed to the cell-free system, rather then to the putative presence of residual whole cells in the cell extracts. Under N_2 atmosphere, methanol was consumed at a rate of 9.4 nmol/min · mg protein during the initial 1 h of the incubation. Isotacho-

phoretic analysis of the reaction mixtures indicated that the relative high rate of methanol conversion with respect to the rate of methane formation was due to the rapid accumulation of CH_3-S-CoM during this stage (data not shown). After prolonged incubation, methanol and methane were consumed and produced, respectively, in a ratio of 1.2:1, which is in fair agreement with the stoichiometry of eqn. 1. Methane formation from methanol, both under N_2 and under H_2 atmosphere, was completely inhibited in the presence of Ti(III)citrate (5-14 mM), which could be attributed to a specific inhibition of the methanol:HS-CoM methyltransferase reaction. Comparison of the specific activities of the various partial reactions involved in methanol conversion under N_2 demonstrated that the rate-limiting step in the process was the oxidation of methanol to the formal redox level of formaldehyde (Table 1).

Methyl-tetrahydromethanopterin synthesis from methanol and tetrahydromethanopterin. Cell-free extracts from *M. barkeri* that had been depleted from low-molecular-weight compounds by extensive washing on an Amicon PM-30 ultrafiltration membrane were capable of 5-methyl-H_4MPT synthesis from methanol and H_4MPT when incubated under H_2 atmosphere in the presence of 5 mM Ti(III)citrate. The product was identified on the basis of its retention time on HPLC, the characteristic UV-visible light absorbance spectrum, and comigration with authentic 5-methyl-H_4MPT (Fig. 3). An average 28% of H_4MPT added became methylated under the reaction conditions employed. This value was already reached immediately after preparation and gassing with H_2 of the reaction mixtures. Prolonged incubation at 37°C did not further increase the amount of 5-methyl-H_4MPT formed, suggesting that the reaction had come to an equilibrium. About half the amount of methyl-H_4MPT was produced, when incubation took place under N_2, whereas maximally only 4-8% H_4MPT was converted after 90 min incubation under H_2 atmosphere in absence of Ti(III)citrate. No other H_4MPT derivatives besides the substrate and methyl-H_4MPT could be detected in the different incubation mixtures. No methyl-H_4MPT was formed in the absence of methanol, H_4MPT, or PM-30-washed extract. In addition, boiled extract (30 min at 100°C) or aerobically stored extract (24 h under air at 4°C) were unable to catalyze methyl-H_4MPT synthesis from H_4MPT and methanol. Addition of HS-CoM (10 mM) or NaCl (20 mM) had no effect on the amount of methyl-H_4MPT produced, when incubation took place in the presence of Ti(III)citrate. ATP (2.4 mM), however, could substitute for the latter compound in its stimulation of the reaction.

Resolution of methanol:H_4MPT methyltransferase activity. After fractionation of PM-30 extract of *M. barkeri* on DEAE-Sepharose

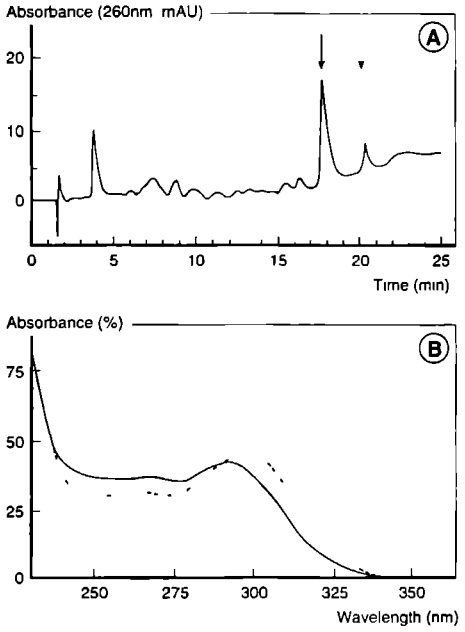

Fig. 3. HPLC-analysis of H_4MPT and its reaction product after incubation with methanol and PM-30 washed extract of *M barkeri* Incubation and HPLC analysis were performed as described under "Materials and Methods" The HPLC elution pattern at 260 nm is shown (A) with the UV-visible absorbance spectra (B) of the peaks at 17 7 min (——) and 20 2 min (- - -) The spectra and retention times of the first (——►) and second (- - -►) peak were identical to those of H_4MPT and 5-methyl-H_4MPT at pH 3, respectively.

methanol:H_4MPT methyltransferase activity was present in the opaque pass-through fractions. The fraction obtained after elution of all bound protein was able to catalyze transfer of the methyl group of methanol to HS-CoM, but was unable to produce methyl-H_4MPT from methanol and H_4MPT. Centrifugation of the opaque pass-through fractions at 100,000 x *g* pelleted all methanol:H_4MPT methyltransferase activity, indicating a membrane association of the enzyme(system) involved. In agreement herewith, by the same procedure methyl-H_4MPT:HS-CoM methyltransferase was enriched 14-fold with a recovery of 94% (P. Daas and D. van Lent, unpublished results). The latter methyltransferase was previously identified as a membrane protein (10) 5,10-Methylene-tetrahydromethanopterin reductase activity was not present in the isolated fraction. The membraneous pellet fraction was unable to produce methane from methanol, under either H_2 or N_2, which excluded the presence of any unbroken cells or vesicles of *M. barkeri*.

Evidence for a role of corrinoids in methyl-H_4MPT synthesis from methanol. Incubation of the pellet fraction with methanol and Ti(III) citrate, in absence of H_4MPT, revealed the formation of a small amount of a methylated corrinoid. The product had the same retention time and UV-visible spectrum as authentic methyl-B_{12}-HBI (Fig. 4). To verify

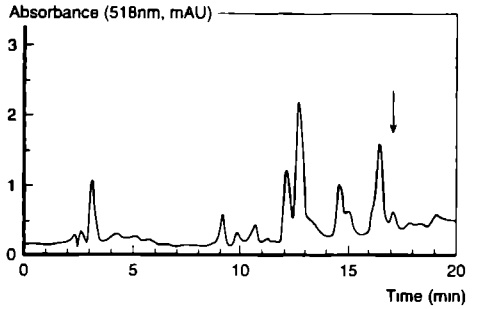

Fig. 4. HPLC-analysis of corrinoids extracted after incubation of methanol with the purified membrane fraction of *M. barkeri*. Purification, incubation, and HPLC analysis were performed as described under "Materials and Methods". The peak at 17.1 min (——►) was identified as methyl-B_{12}-HBI. The peak at 15.0 min could be attributed to aquo-B_{12}-HBI.

that methanol was indeed the source of the methyl group of the corrinoid we repeated the experiments by using [^{14}C]methanol. Autoradiography of the corrinoids extracted revealed that label was incorporated when [^{14}C]methanol was present (data not shown). The radioactive spot had a R_f value of 0.47, which was identical to that of authentic methyl-B_{12}-HBI.

DISCUSSION

Cell-free extract of *M. barkeri* was able to produce methane from methanol under N_2, indicating that part of the methyl groups of methanol were oxidized to allow reduction of the other methyl groups of methanol to methane. The first intermediate observed in the oxidative pathway was 5-methyl-H_4MPT. Synthesis of methyl-H_4MPT from methanol and H_4MPT was specifically brought about by a purified membrane fraction, which was devoid of methanol:HS-CoM methyltransferase activity. The reaction was independent of the presence of HS-CoM and did not strictly require ATP. This excluded an oxidation of methanol via the MT_1/MT_2 system or a methylated MT_1 intermediate (routes II and III in Fig. 1, respectively). Since, the membrane fraction did not contain 5,10-methylene-tetrahydromethanopterin reductase activity and no other H_4MPT derivatives were ever observed besides 5-methyl-H_4MPT, synthesis of methyl-H_4MPT *via* methylene-H_4MPT,

as illustrated in route I of Fig. 1, may also be excluded. The results are in agreement with a direct transfer of the methyl group of methanol to H_4MPT catalyzed by a membrane-bound, oxygen sensitive enzyme-(system). We only observed a partial methylation of H_4MPT under the optimal reaction conditions conforming the expected unfavorable thermodynamics of the reaction ($\Delta G^{\circ'}$ =+2.2 kJ/mol). From a comparison of the overall and partial reactions it was concluded that the rate-limiting step in methanol oxidation was the conversion of methanol to the formaldehyde (5,10-methylene-H_4MPT) level (Table 1). This now seems to be a two step process: the synthesis of 5-methyl-H_4MPT followed by its oxidation to 5,10-methylene-H_4MPT. By the HPLC analysis method used it was not possible to estimate the specific activity of the former step. Consequently it cannot be decided, whether the rate limitation is related to the methyltransferase reaction or that the low concentrations of 5-methyl-H_4MPT formed limit the oxidation rate of 5,10-methylene-H_4MPT in our cell-free system used.

Methyl group transfer reactions in anaerobic organisms including methanogens are frequently catalyzed by extremely oxygen-sensitive corrinoid proteins that require an ATP and/or Ti(III)citrate-dependent reductive activation (1,18,25). The observation that both compounds stimulated 5-methyl-H_4MPT synthesis suggests a role of a corrinoid protein in the reaction studied here. The findings that methyl-B_{12}-HBI, albeit in low amounts, could be detected by HPLC upon incubation of the membrane fraction with methanol and that labeled methyl-B_{12}-HBI was formed with [^{14}C]methanol provide direct evidence for a role of a corrinoid protein. In the membranes of *M. barkeri* the corrinoid-containing 5-methyl-H_4MPT:HS-CoM methyltransferase (10, this paper) is present. The enzyme catalyzes the reversible methylation of its B_{12}-HBI prosthetic group with 5-methyl-H_4MPT and the subsequent -essentially irreversible- transfer of the methyl group from methyl-B_{12}-HBI to HS-CoM, the whole being connected to sodium translocation out of the cell (7,9). Though this seems not very likely to us, on the basis of our findings it cannot be ruled out that the enzyme has even one more function: the activation of methanol to form methyl-B_{12}-HBI. Alternatively, 5-methyl-H_4MPT synthesis from methanol may be catalyzed by a novel (corrinoid) enzyme. Results of the studies by Müller et al. (7,9) predict that this should be a sodium-translocating protein as well. It will be clear that a further resolution of the membrane system will be required to give an answer to these points.

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Concluding remarks

Abbreviations used: Co/Fe-S protein, corrinoid/iron-sulfur protein; coenzyme F_{420}, a 8-hydroxy-5-deazaflavin derivative; CoM-S-S-HTP, the heterodisulfide of coenzyme M and 7-mercaptoheptanoylthreonine phosphate; HS-CoM, coenzyme M, 2-mercaptoethanesulfonic acid; H_4SPT, 5,6,7,8-tetrahydrosarcinapterin; MAP, methyltransferase activation protein; MT_1, methanol:5-hydroxybenzimidazolylcobamide methyltransferase; MT_2, Co-methyl-5-hydroxybenzimidazolylcobamide: HS-CoM methyltransferase; MTZ, metronidazole.

In this chapter three subjects are reviewed. First, the two notable differences in our study of the methanol:coenzyme M methyltransferase reaction as compared to the work of van der Meijden et al. [15] are discussed. Next, the activation mechanism of methanol:5-hydroxybenzimidazolylcobamide methyltransferase (MT_1) is evaluated. In the final section, this mechanism is compared with the activation mechanisms of other methanogenic methyl group transfer-catalyzing enzymes.

8.1. Differences with the work of van der Meijden et al.

In our hands four proteins are required for transfer of the methyl group of methanol to coenzyme M (HS-CoM). Two of these components are responsible for the overall transfer of the methyl moiety, viz. methanol:5-hydroxybenzimidazolylcobamide methyltransferase (MT_1) and Co-methyl-5-hydroxybenzimidazolylcobamide: HS-CoM methyltransferase (MT_2). MT_1 was purified before by van der Meijden et al. to approximately 90% homogeneity with a purification factor of 2.8 on the basis of the specific activity and a purification factor of 5.7 on the basis of the B_{12}-HBI content per mg of protein [17]. They found 3.4 mol of B_{12}-HBI per mol of protein [17]. It is the corrinoid content that is not confirmed by us [Chapter 6]. We repeatedly purified large quantities of MT_1 to 100% homogeneity, with a purification factor of 33 (on the basis of the specific activity), from various batches of cell extract and always obtained an amount of about 1.7 ($\pm$ 0.4) mol B_{12} per mol of protein. This is only half of the amount reported by van der Meijden et al. [17]. Since they only purified MT_1 once to 90% purity, we believe that our (reproducible) data from the 100% pure protein is much more reliable.

The amount of MT_1 present in cell extract varied, depending on the batch used, between 2.9 and 10% of the total amount of protein [P. Daas, unpublished results]. Van der Meijden et al. reported an amount of about 15% [17]. Perhaps these varying amounts of MT_1 reflect small differences in growth phase of *Methanosarcina barkeri* during harvesting of the cells. This could point to some regulatory mechanism of MT_1 synthesis during growth. All in all, the body of the work of van der Meijden et al. corroborated well with our results.

8.2. Reductive activation of MT_1

MT_1 is only able to accept the methyl group of methanol when the central cobalt atom of its corrinoids are in their fully reduced Co(I) state [16]. This so-called reductive activation results from the combined action of H_2, hydrogenase, Methyltransferase Activation Protein (MAP), and ATP [Chapter 2]. Ferredoxin is not absolutely required but it stimulates the apparent reaction rate of methyl group transfer [Chapter 2]. These components activate MT_1 by (i) producing reducing equivalents for the actual reduction of the cobalt atom of the corrinoids (H_2, hydrogenase, ferredoxin) and by (ii) facilitating the reduction of the cob(II)amide of MT_1 (MAP, ATP) [Chapter 6]. The action of the reducing system and the cob(II)amide modifying system will be discussed below. All reactions involved in the transfer of the methyl group from methanol to coenzyme M and the activation of MT_1 are presented in Fig. 1.

8.2.1. The reducing system

H_2 and hydrogenase are able to catalyze the one-electron reduction of the cob(III)amide and the "base-off" coordinated cob(II)amide of MT_1 to the "base-on" cob(II)amide and the cob(I)amide of MT_1, respectively [Chapter 6]. Ferredoxin enhances one or both of these reductions [Chapter 2]. In *M. barkeri* two distinct hydrogenases are present: a high-molecular-weight coenzyme F_{420}-dependent hydrogenase [2], and a non-F_{420}-dependent hydrogenase of lower molecular weight [9]. In the presence of the artificial electron acceptor metronidazole (MTZ) the methanol:HS-CoM methyltransferase reaction is inhibited to a large extent [4]. A maximum velocity of methyl transfer is established after MTZ has been fully (and irreversibly) reduced. Under H_2-atmo-sphere, MTZ can not be reduced by hydrogenase directly, but specific-ally requires the mediation of ferredoxin or flavodoxin [1]. In view of the fact that MT_1 does not contain iron-sulfur clusters [Chapter 6] reduction of MTZ must be catalyzed by the hydrogenase-ferredoxin couple. Since only the F_{420}-dependent hydrogenase is able to reduce ferredoxin [2,10], it is evident that this type of hydrogenase is able to donate electrons for the reduction of the cobalt atom of the corrinoids of MT_1. A role for the non-F_{420} reducing hydrogenase in this activation process seems unlikely [9,10], but can not be fully excluded as methyl transfer is not fully inhibited by MTZ [4] and activation of MT_1 is possible in absence of ferredoxin [Chapter 2].

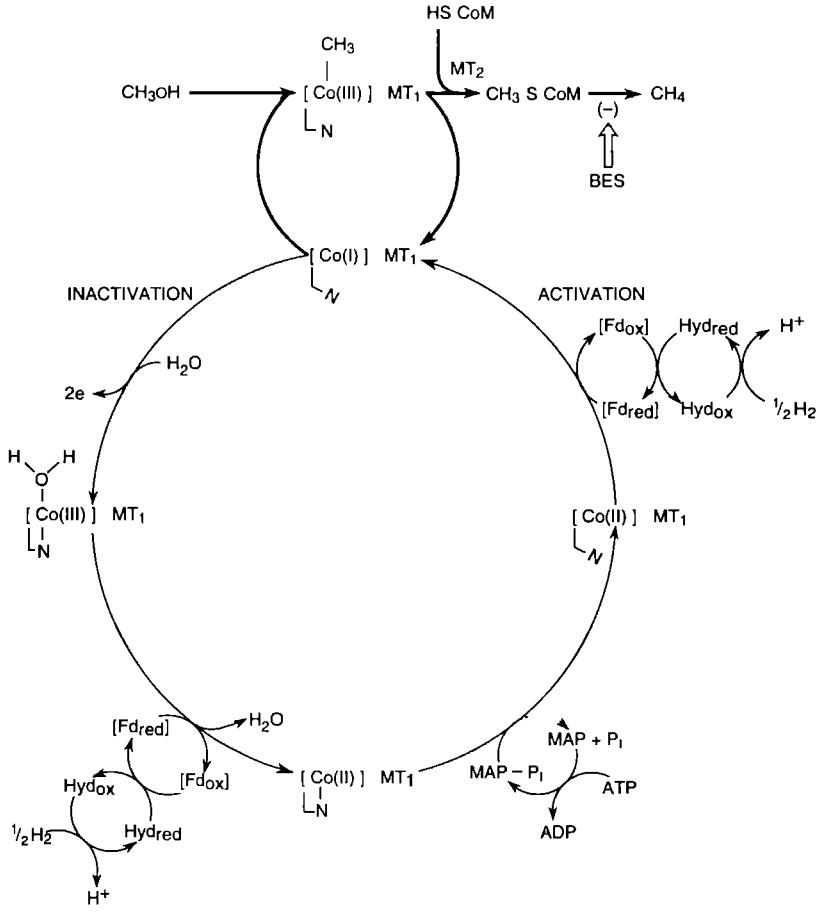

Fig. 1. Carbon and electron flow scheme proposed for transfer of the methyl group from methanol to coenzyme M in *Methanosarcina barkeri* The overall methyltransferase reaction is drawn in bold lines The sequence of reactions involved in the reactivation of methanol 5-hydroxybenzimidazolylcobamide methyltransferase (MT_1) is indicated by the thin lines [Co(III)], [Co(II)], and [Co(I)] represent the various oxidation states of the cobalt of the corrinoid prosthetic groups of MT_1 Coordination of N-3 of the 5-hydroxybenzimidazolyl base is illustrated by the connecting line between Co and N Because the coordination of the base is unknown for the methylated corrinoids of MT_1 a dashed line is drawn here In the 'base off' Co(II) state no ligand is shown but it is possible that a ligand with poor delocalization properties, e g water, is present The dashed line used for the dephosphorylation of MAP-phosphate indicates that the exact sequence of events for this particular reaction is not fully established BES, 2 bromoethanesulfonic acid, Fd, ferredoxin, HS CoM, coenzyme M, Hyd, hydrogenase, MT_2, Co-methyl 5-hydroxybenzimidazolylcobamide HS CoM methyltransferase Ferredoxin is shown in parenthesis because activation can occur in absence of this protein

8.2.2. The cob(II)amide modifying system

In absence of MAP or ATP reduction of the base-on coordinated Co(II) corrinoids of MT_1 is impossible. Both components are required for the conversion of "base-on" into "base-off" cob(II)amides of MT_1 [Chapter 6]. In non-protein bound corrinoids the "base-off" form is much easier to reduce then the base-coordinated form [Chapter 4]. In an identical way, it is conceivable that MAP and ATP enable the reduction of the cob(II)amide of MT_1 by electrons derived from hydrogen. Since MAP interacts with ATP during the activation of MT_1 and is phosphorylated by the terminal phosphate of ATP [Chapter 5] it is suggested that phosphorylated MAP is the actual effector in the conversion of "base-on" to "base-off" Co(II) MT_1 [Chapter 6]. Whether, the phosphate of phosphorylated MAP is actually transferred to MT_1 during this process is unknown. But it is evident that MAP must be dephosphorylated eventually, for it is isolated in a form which is fully inactive in absence of ATP [Chapter 2].

8.3. Activation of methanogenic methyltransferases

When all results, known to date, with respect to the effect of ATP, MAP, and Ti(III)citrate on the methyl group transfer reactions catalyzed by *M. barkeri* and *Methanobacterium thermoautotrophicum* are combined, an intriguing phenomenon is revealed. From the data presented in Table 1 it is clear that there exist, at least, three different mechanisms for the reductive activation of methyltransferases in methanogens.

I) An activation mechanism which strictly requires ATP and involves the action of MAP. This system is involved in the activation of MT_1 and probably also in the activation of the enzymes concerned with methyl group transfer from tri-, di-, and monomethylamine to HS-CoM.

II) An activation mechanism which is completely independent of ATP. The activation of the corrinoid/iron-sulfur (Co/Fe-S) protein involved in methyl group transfer from acetyl-coenzyme A to tetrahydrosarcinapterin (H_4SPT) is the best studied example of this mechanism.

Table 1. *Influence of ATP, MAP, and Ti(III)citrate on methanogenic methyltransferase reactions.*

Methyltransferase reaction		Effect of ATP	Necessity for ATP	Effect of MAP	Effect of Ti(III)	References
Methanosarcina barkeri						
CH_3OH	: HS-CoM	+[a]	+	+	–	[Chap.2,18]
CH_3NH_2	: HS-CoM	+	(+)	?	?	[14][b]
$(CH_3)_2NH$	: HS-CoM	+	+	+	?	[14][b]
$(CH_3)_3N$	: HS-CoM	+	+	+	?	[13,14][b]
CH_3-H_4SPT	: HS-CoM	+	–	–	+	[19,21,22]
CH_3OH	: H_4SPT	+	–	–	+	[Chap.7]
CH_3-CO-CoA	: H_4SPT	–	–	–	?	[5,6,20]
Methanobacterium thermoautotrophicum[c]						
CH_3-H_4MPT	: HS-CoM	+	–	–	+	[3,11]

[a] Explanation of the signs used: +, stimulation; –, no effect; ?, unknown. Brackets are used to indicate results obtained from partially resolved systems.

[b] Includes unpublished results of Roel Wassenaar, with permission.

[c] Here CoM-S-S-HTP also stimulates the methyltransferase reaction [12]. Identical result were obtained for strain strain ΔH and Marburg. MAP is not involved because the protein is not present in these organisms [P. Daas, unpublished results].

III) An activation mechanism which is stimulated by, but not absolutely dependent on, ATP and does not involve the action of MAP. Here, Ti(III)citrate is able to substitute for the activation system. The membrane-bound methyl-H_4SPT: coenzyme M methyltransferase of *M. barkeri* and the corresponding enzymes of *M. thermoautotrophicum* are representatives of this group. It should be noted, however, that in the latter organism the heterodisulfide of HS-CoM and 7-mercaptoheptanoylthreonine phosphate (CoM-S-S-HTP) also stimulates methyl group transfer [12] which is not the case in *M. barkeri* [7]. The methanol:H_4SPT methyltransferase from *M. barkeri* [Chapter 7] also belongs to this group.

Since acetate grown *M barkeri* is able to convert methanol as well [8], this organism is an example of an archaeon which contains methyltransferases representing all of the three groups described above, viz MT_1, the Co/Fe-S protein of carbon monoxide dehydrogenase, and methyl-H_4SPT HS-CoM methyltransferase The three distinguishable activation mechanisms can thus be collectively present in a single organism. Methanogens are still a surprising microbial group !

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Summary/Samenvatting

SUMMARY

Methanogens are obligate anaerobic micro-organisms which obtain their energy for growth from the conversion of a limited number of one-carbon compounds or acetate to methane. In this thesis the conversion of methanol to methane and CO_2, by the methylotrophic methanogenic archaeon *Methanosarcina barkeri*, is described.

In chapter 1 an introductory review is given of methane-producing archaea and of the biochemistry of the process of methanogenesis from methanol, H_2/CO_2, and acetate, as catalyzed by *Methanosarcina* species. The role of corrinoids, their function in methyl group transfer, and the methyltransferase reactions of methanogenesis are extensively discussed. Corrinoid-dependent methyltransferase reactions in non-methanogens are also briefly reviewed.

The main part of this thesis is concerned with the transfer of the methyl group from methanol to coenzyme M. As a result, the methylated form of coenzyme M is produced which is the final substrate for methanogenesis in all methanogens studied so far. From previous studies it was known that the transfer of the methyl group of methanol to coenzyme M was catalyzed by the concerted action of two methyltransferases. The first methyltransferase, methanol:5-hydroxy-benzimidazolylcobamide (B_{12}-HBI) methyltransferase (MT_1), binds the methyl group of the substrate to its corrinoid prosthetic group. Next, the methyl group of the methylated corrinoid-containing protein is transferred to coenzyme M by Co-methyl-B_{12}-HBI:coenzyme M methyltransferase (MT_2). MT_1 is only able to bind the methyl group of methanol when the central cobalt atom of its corrinoid prosthetic group is present in the fully reduced Co(I) state. Upon isolation of MT_1 or even during catalysis the enzyme becomes inactivated as the result of Co(I) oxidation. Reactivation is possible and requires H_2, hydrogenase, ferredoxin, and ATP.

Chapter 2 reports the enhanced resolution of the methanol:coenzyme M methyltransferase system of *Methanosarcina barkeri*. When cell extract of *Methanosarcina barkeri* was submitted to DEAE-Sepharose ion-exchange chromatography one additional protein was found to be required for the overall methyltransferase reaction. The protein was shown to be specifically needed for the reductive activation of MT_1 and was called Methyltransferase Activation Protein (MAP). Although, ferredoxin stimulated the apparent reaction rate of methyl group transfer, it appeared not to be absolutely required.

In chapter 3 the purification and characterization of the ferredoxin that facilitates the methanol:coenzyme M methyltransferase reaction is described. It was isolated as a dimer with 2[4Fe-4S] clusters per 6.2-kDa subunit. The midpoint redox potential of the $[4Fe\text{-}4S]^{2+;1+}$ couples was -322 mV (21°C, pH 7) and was temperature- but not pH-dependent.

The electrochemistry of B_{12}-HBI was studied in chapter 4. The (acidic) dissociation constants and standard oxidation-reduction potentials of the Co(III)/Co(II) and Co(II)/Co(I) couples of B_{12}-HBI were measured and compared to those described for cobalamin, the ubiquitously corrinoid in nature. This showed that the redox potentials of the 5-hydroxybenzimidazolyl (HBI) containing cobamide (B_{12}-HBI) were almost identical to those of the 5,6-dimethylbenzimidazolyl (DMBI) containing cobamide (cobalamin). The HBI-base, however, proved to be the weaker ligand, thus favoring dissociation of the nucleotidic base. This seemed to suggest the importance of "base-on"-to-"base-off" conversions in methanogenic transmethylation reactions.

Chapter 5 reports the purification and characterization of MAP. The protein, isolated as a monomer of 60 kDa, interacted with ATP during the activation of MT_1. The purified protein became readily phosphorylated by the terminal phosphate group of ATP. This suggested that phosphorylated MAP is the actual effector in the activation of MT_1.

The mechanism of the reductive activation of MT_1 was unraveled in chapter 6. Here, it was shown that H_2, hydrogenase, and ferredoxin were able to catalyze the one electron reduction of the cob(III)amide and "base-off" cob(II)amide of MT_1 to the "base-on" cob(II)amide and cob(I)amide, respectively. MAP and ATP were required for the conversion of the "base-on" cob(II)amide of MT_1 to the "base-off" form. In non-protein bound cob(II)amide this resulted in an increase of the midpoint redox potential of the Co(II)/Co(I) couple from -592 to -500 mV (Chapter 4). In an identical way, the action of MAP and ATP could facilitate the reduction of the cob(II)amide of MT_1 by electrons derived from H_2 (-414 mV).

The presence of methanol:5,6,7,8-tetrahydromethanopterin methyltransferase activity in *Methanosarcina barkeri* is described in chapter 7. This reaction was the first step in the oxidation of methanol to CO_2 and was specifically catalyzed by a membrane bound protein.

In chapter 8 the results presented in this thesis are discussed and compared with the results of studies of other methanogenic methyltransferase reactions. A comparison between the reductive activation mechanisms of these methyltransferase reactions revealed that there are, at least, three different ways whereupon methanogenic methyltransferases could be activated.

Methaanbacteriën zijn strikt anaërobe micro-organismen die kunnen leven van de omzetting van een beperkte groep C_1-bevattende substraten of acetaat naar methaan. In dit proefschrift wordt de omzetting van methanol naar methaan en CO_2, door het methylotrofe methanogene archaeon *Methanosarcina barkeri*, beschreven.

In hoofdstuk 1 worden de methaanproducerende archaea geïntroduceerd en wordt de biochemie van het proces van methaanvorming uit methanol, H_2/CO_2 en acetaat, door *Methanosarcina* soorten, behandeld. Uitvoerig wordt ingegaan op de functie van corrinoïden, hun belang bij de overdracht van methylgroepen en de methyltransferase reacties betrokken bij de vorming van methaan. De corrinoid-afhankelijke methyltransferase reacties van niet-methanogenen worden ook kort besproken.

Het grootste gedeelte van dit proefschrift behandelt de overdracht van de methylgroep van methanol naar coenzym M. Deze reactie leidt tot de vorming van gemethyleerd coenzym M dat het uiteindelijke substraat is voor de vorming van methaan bij alle, tot nu toe, bestudeerde methaanbacteriën. Uit eerder uitgevoerd onderzoek was reeds bekend dat de overdracht van de methylgroep van methanol naar coenzym M wordt gekatalyseerd door twee methyltransferases. Het eerste enzym, methanol:5-hydroxybenzimidazolylcobamide (B_{12}-HBI) methyltransferase (MT_1), bindt de methylgroep van methanol aan zijn corrinoid prosthetische groep. Daarna kan de methylgroep van dit gemethyleerd corrinoid-bevattende eiwit worden overgedragen naar coenzym M door het Co-methyl-B_{12}-HBI:coenzym M methyltransferase (MT_2). MT_1 kan alleen de methylgroep van methanol binden wanneer het centrale cobalt atoom van zijn corrinoid prosthetische groep zich in de meest gereduceerde Co(I) toestand bevindt. Na zuivering van MT_1, of zelfs gedurende de methyltransferase reactie, inactiveert het enzym door oxydatie van het Co(I) atoom. Reactivatie is mogelijk en vereist de aanwezigheid van H_2, hydrogenase, ferredoxine en ATP.

Hoofdstuk 2 beschrijft de verbeterde scheiding van het methanol: coenzym M methyltransferase systeem van *Methanosarcina barkeri*. Wanneer celvrij extract van dit organisme gescheiden werd met behulp van een DEAE-Sepharose anionenwisselaar werd één extra eiwit gevonden dat noodzakelijk was voor de overdracht van de methylgroep van methanol naar coenzym M. Aangezien dit eiwit specifiek betrokken bleek bij de reductieve activering van MT_1 werd het methyltransferase activerings eiwit genoemd, -in het Engels- afgekort als MAP. De

aanwezigheid van ferredoxine stimuleerde de methyltransferase reactie duidelijk maar het bleek niet absoluut noodzakelijk te zijn.

In hoofdstuk 3 wordt de zuivering en karakterisering van het ferredoxine dat stimulerend werkt op de methanol:coenzym M methyltransferase reactie besproken. Het eiwit werd gezuiverd als een dimeer met 2[4Fe-4S] clusters per subunit van 6.2 kDa. De redoxpotentiaal van het $[4Fe\text{-}4S]^{2+,1+}$ koppel was -322 mV (21°C, pH 7) en was temperatuur- maar niet pH-afhankelijk.

De electrochemie van B_{12}-HBI werd bestudeerd in hoofdstuk 4. De (zure) dissociatie constanten en standaard oxydatie-reductie potentialen van de Co(III)/Co(II) en Co(II)/Co(I) koppels van B_{12}-HBI werden bepaald en vergeleken met die van cobalamine, het in de natuur meest voorkomende corrinoide. Hieruit volgde dat de redoxpotentialen van het 5-hydroxybenzimidazolyl (HBI) bevattende cobamide (B_{12}-HBI) vrijwel identiek waren aan die van het 5,6-dimethylbenzimidazolyl (DMBI) bevattende cobamide (cobalamine). De HBI-base bleek echter een zwakkere ligand te zijn, waardoor B_{12}-HBI een lichte voorkeur voor dissociatie van het nucleotide heeft. Dit zou mogelijk kunnen wijzen op het belang van de omzetting van base gecoördineerd ("base-on") naar niet-base gecoördineerd ("base-off") B_{12}-HBI tijdens methanogene methyltransferase reacties.

Hoofdstuk 5 beschrijft de zuivering en karakterisering van MAP. Dit eiwit, dat bestond uit een monomeer van 60 kDa, werkte samen met ATP gedurende de activering van MT_1. Het gezuiverde eiwit bleek door de eindstandige fosfaatgroep van ATP gefosforyleerd te worden. Dit leek erop te wijzen dat gefosforyleerd MAP de feitelijke effector was tijdens de activering van MT_1.

Het mechanisme van de reductieve activering van MT_1 werd opgehelderd in hoofdstuk 6. Hierin werd aangetoond dat H_2, hydrogenase en ferredoxine in staat waren de één-electron reductie van het cob(III)amide en het "base-off" cob(II)amide van MT_1 naar, respectievelijk, het "base-on" cob(II)amide en het cob(I)amide te katalyseren. MAP en ATP bleken noodzakelijk te zijn voor de omzetting van de "base-on" vorm van het cob(II)amide van MT_1 naar de "base-off" vorm. Bij het niet-eiwit gebonden cob(II)amide resulteerde dit in een toename van de redoxpotentiaal van het Co(II)/Co(I) koppel van -592 mV naar -500 mV (hoofdstuk 4). Op een soortgelijke manier zou de reductie van het cob(II)amide van MT_1, door electronen afkomstig van H_2 (-414 mV), vergemakkelijkt kunnen worden door de werking van MAP en ATP.

De aanwezigheid van methanol:5,6,7,8-tetrahydromethanopterine methyltransferase activiteit in *Methanosarcina barkeri* wordt behandeld

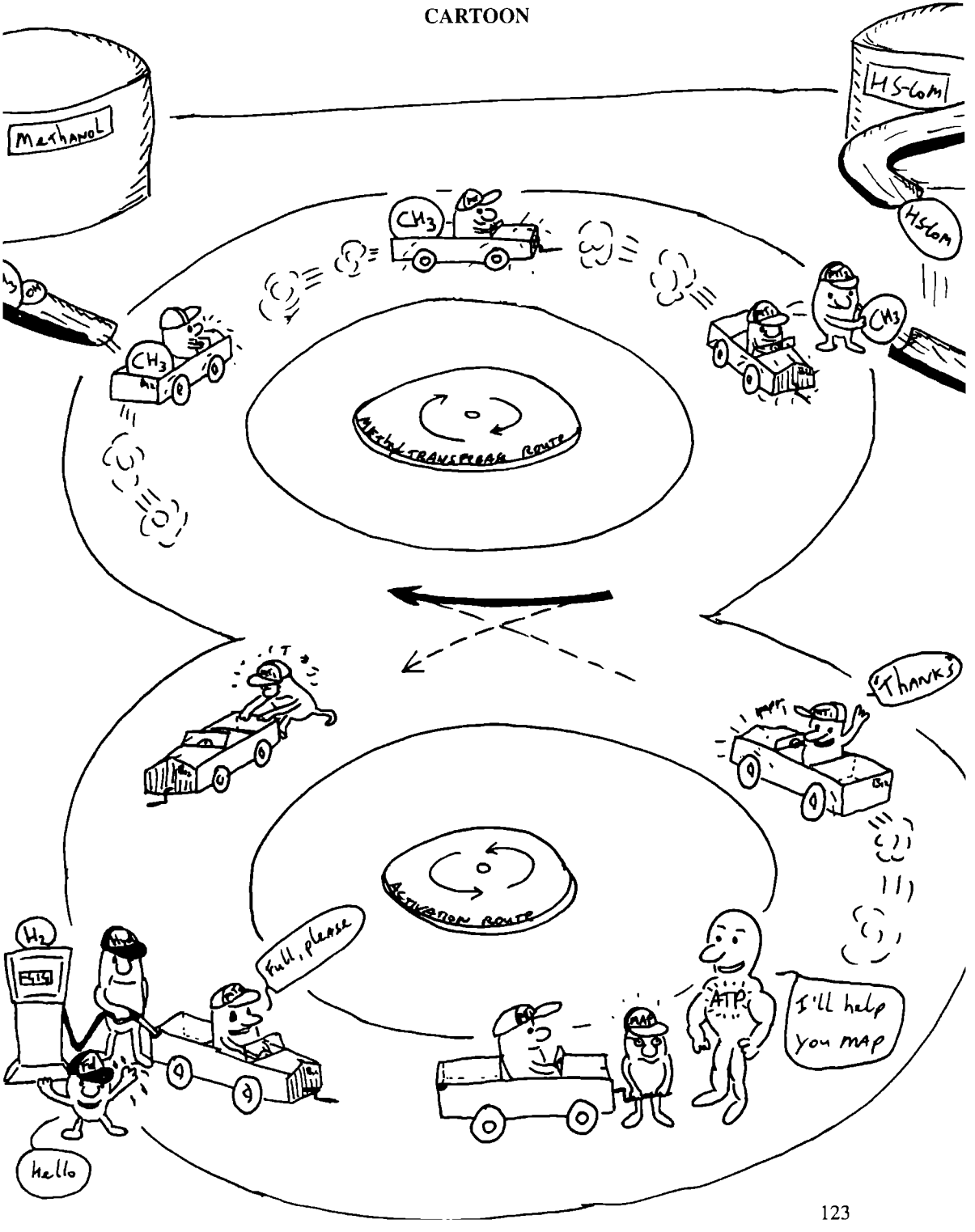
in hoofdstuk 7. Deze reactie is de eerste stap in de oxydatie van methanol naar CO_2 en werd specifiek gekatalyseerd door een membraan gebonden eiwit.

In hoofdstuk 8 worden de bevindingen van dit proefschrift bediscussieerd en vergeleken met de resultaten van studies van andere methanogene methyltransferase reacties. Een vergelijking tussen de mechanismen van reductieve activering voor deze methyltransferase reacties leerde dat er, wellicht, drie verschillende manieren zijn waarop methanogene methyltransferases geactiveerd kunnen worden.

Cartoon of the methanol:coenzyme M methyltransferase reaction, illustrating the function of hydrogen, hydrogenase, ferredoxin, methyltransferase activation protein (MAP), and ATP in the activation of methanol:5-hydroxybenzimidazolylcobamide methyltransferase (MT_1).

Cartoon van de methanol:coenzyme M methyltransferase reactie waarin de functie van waterstof, hydrogenase, ferredoxine, methyltransferase activerings eiwit (MAP) en ATP bij de activering van het methanol: 5-hydroxybenzimidazolylcobamide methyltransferase (MT_1) duidelijk wordt gemaakt.

Abbreviations used: ATP, adenosine triphosphate; B_{12}, 5-hydroxybenzimidazolylcobamide; CH_3, methyl group; CH_3OH, methanol; Fd, ferredoxin; H_2, hydrogen; Hyd, hydrogenase; HS-CoM, coenzyme M, 2-mercaptoethanesulfonic acid; MAP, methyltransferase activation protein; MT_1, methanol:5-hydroxybenzimidazolylcobamide methyltransferase; MT_2, Co-methyl-5-hydroxybenzimidazolylcobamide: HS-CoM methyltransferase.


Methanol
HS-CoM
CH_3
Methyl-transferase route
Activation route
H_2
Full, please
Hello
ATP
I'll help you MAP
Thanks

Dit proefschrift zou niet tot stand zijn gekomen zonder de hulp van velen. Al die personen (of ze nu wel of niet hieronder met naam worden genoemd) wil ik van harte bedanken voor hun hulp, steun, ideeën en suggesties.

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Piet

Piet J.H. Daas werd geboren op 6 mei 1963 te Oudenbosch. In 1979 behaalde hij het diploma MAVO-4 aan de Pius XII Mavo te Oudenbosch. Via een voorbereidend jaar HBO slaagde hij in 1982 voor het diploma HBO-A, in de medisch microbiologische richting, aan het Dr. Struycken Instituut te Etten-leur. In datzelfde jaar werd hij door de krijgsmacht wegens "hooikoorts" ongeschikt geacht voor het vervullen van de dienstplicht. Doordat de HBO-opleiding zijn interesse aanwakkerde voor de biologie, en de microbiologie in het bijzonder, besloot hij in 1983 tot het volgen van een Colloquium Doctum voor de studierichting Biologie aan de Katholieke Universiteit Nijmegen (direct doorstromen van het HBO naar de Universiteit was toen nog niet mogelijk).

In 1984 werd hij toegelaten tot de studie Biologie aan de Katholieke Universiteit Nijmegen, waarna het propaedeutisch examen in 1985 behaald werd. Het doctoraal programma omvatte een uitgebreid bijvak Exobiologie (Prof. Dr. A.W. Schwartz en Dr. G.C. Bakker), waarbij onderzoek werd gedaan naar de mogelijke prebiotische synthese routes van 8-gesubstitueerde nucleoside achtige adenines, en een zeer uitgebreid hoofdvak Microbiologie (Prof. Dr. Ir. G.D. Vogels en Dr. J.T. Keltjens). Tijdens het hoofdvak werden de activering van het B_{12}-bevattende methyltransferase van *Methanobacterium thermoautotrophicum* en de reductieve dehalogenering van chloroform door celvrije extracten van dat organisme onderzocht. Ondanks verwoede pogingen van de toenmalige minister van onderwijs (Deetman) om het afronden van zijn studie te verhinderen lukte het Piet in februari 1990 het doctoraal examen Biologie 'cum laude' te behalen. In die periode werden tevens het diploma Deskundigheid Stralingshygiëne niveau 3 en het diploma Veilige Microbiologische Technieken behaald. Voor zijn studieprestaties ontving hij in 1990 de Unilever Research Prijs.

Van april 1990 tot april 1994 was hij werkzaam als Assistent In Opleiding op het Laboratorium voor Microbiologie van de Katholieke Universiteit Nijmegen waarbij hij "de biochemie van methylgroep overdracht reacties in *Methanosarcina barkeri*" onderzocht. Gedurende het laatste jaar van zijn AIO-periode werd samengewerkt met de afdeling Biochemie aan de Landbouw Universiteit Wageningen. In die periode werd tevens het AIO-onderwijs programma volledig afgerond. Tijdens het schrijven van zijn proefschrift zette hij de, via Internet te bereiken, World-Wide-Web pagina's van het Laboratorium voor de Microbiologie op (http://www-micrbiol.sci.kun.nl/micrbiol/).

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